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# Liver Transcriptomic Analysis After Short- and Long-term Feeding of Soy Protein Isolate and Its Ability to Reduce Liver Steatosis in Obese Zucker Rats

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# Liver Transcriptomic Analysis After Short- and Long-term Feeding of Soy Protein Isolate and Its Ability to Reduce Liver Steatosis in Obese Zucker Rats

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

Melisa Kozaczek
Universidad Nacional de Misiones (UNAM)
Bachelor of Science in Genetics, 2014

May 2020
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

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## Abstract

According to the Centers for Disease Control and Prevention (CDC) the prevalence of obesity in adults in the United States during 2017-2018 was a 42.4%, a high number considering all the risks factors associated with this disorder, such as cardiovascular disease, insulin resistance, diabetes type 2, and fatty liver disease, among others. Fatty liver disease is the accumulation of lipids in the liver that can account for more than 5 to 10% of the liver's weight. There are two types of fatty liver disease, alcoholic fatty liver disease (AFLD), and non-alcoholic fatty liver disease (NAFLD). AFLD is the detrimental accumulation of lipids in the liver due to sustained alcohol consumption. NAFLD is the aggregation of lipids in the hepatocytes that cannot be explain by alcohol intake. In this study, we analyzed the beneficial impact of consuming a soy-based diet in ameliorating the effects of NAFLD on obese Zucker rats.

We conducted global gene expression analysis on samples extracted from livers of Zucker rats that were fed diets containing either soy protein isolate (SPI) or casein (CAS) during 8 (short-term) and 16 weeks (long-term) (Hakkak et al. 2015). In order to validate the transcriptomics data we run qPCR on some of the most deferentially expressed genes, and found good correlation between both using a cut off value of 1.3 fold and $P < 0.005$. There were several genes either up- or down-regulated in SPI feeding group that are consistent with the literature. There were also novel findings linking the up regulation of a gene (such as NPTX2) with SPI and NAFLD that were never reported before to our knowledge. In addition, we used Ingenuity Pathway Analysis (IPA) software to help us to interpret the data analysis. We compared the effects of the short-term SPI diet versus long-term on the same diet. The results seem to indicate that the longer the obese rats were on the SPI diet the more beneficial were its effects in two main functions we focused on: inflammatory response predicted by IPA to be inhibited, and lipid metabolism, predicted to be activated in SPI feeding versus the control group.

Acknowledgements

There are many people that helped me and guided me to transit the path of research in biological sciences. In first place, I would like to thank my advisor, Dr. Walter Bottje, for his wise advice and infinite patience, always. Also, I would like to thank Dr. Kentu Lassiter and Dr. Elizabeth Green for their help with the techniques required in the laboratory. In addition, I would like to specially thank my co-advisor, Dr. Hakkkak, the pioneer of this research, for his confidence, encouragement, and for always being there for me even from afar. To my committee. I would like to thank also to my first advisor here at the University of Arkansas, Dr. Andrew Alverson, who received me in his diatoms lab and initiated me on the course of becoming a graduate student. To Dr. Alverson's crew members, Dr. Teofil Nakov and Dr. Elizabeth Ruck, whom through daily work and friendship transmitted me general wisdom in life. To Dr. Jeffrey Mower at the University of Lincoln-Nebraska for giving me the opportunity to work in his lab during a very enlightening summer in 2014, which finally led me to the decision to come to the US to specialize in the biology field. Finally, to my firsts advisor and co-advisor ever, Dr. Rosalia Paz and Dr. Virginia Sanchez-Puerta, for helping me to give the firsts steps in the fascinating world of plant genome research, which opened not only my eyes but many doors in my professional life.

I would like to thank to the University of Arkansas, to the Center of Excellence for Poultry Science Department, to the Arkansas Biosciences Institute, and the Graduate School.

Mostly, I would like to thank my family. This study is dedicated to them in many ways. To my beloved husband Caleb, for his daily unconditional love and encouragement. To my sisters Estefania and Joana, the best friends life gave me, whom despite the separation of years and the distance for the first time, always supported me and inspired me. To my father, Luis, and most importantly to my mother, Gloria, my first inspiration, for loving me and giving me life. To my friends, the brothers and sisters I chose for life and stayed with me forever, no matter what:

Mayte, Romina, Oscar, Mara, Debora, Noemi, charming Elizabeth, and Diyana. Special mention deserve Milo, Samira and Isabella, for enlighten my days.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

An important medical condition linked to metabolic syndrome and obesity, nonalcoholic fatty liver disease (NAFLD) is characterized by mild steatosis, that if left unchecked, leads to non-alcoholic steatohepatitis (NASH), and finally to cirrhosis if damage continues. NAFLD can be described as abnormal accumulation of lipids within liver cells not associated with alcohol consumption. It is commonly accepted that the course of NAFLD takes place in two stages. In the first stage, insulin resistance develops that is accompanied by lipid accumulation in the liver in the form of triglycerides. In the second stage, mitochondrial dysfunction with mitochondrial reactive oxygen species production promotes oxidative stress leading to inflammation and hepatic fibrosis (Berardis, 2014; Fang, 2018). A revision of the progression of pathogenesis has been proposed in which NAFLD can be tentatively diagnosed in patients with elevated liver enzymes and by imaging in the absence of other causes of liver disease. However, a definitive diagnosis can only be made by liver biopsy (Serviddio et al., 2008; Weiß et al., 2014). Estimations of the prevalence suggest that NAFLD could be the most common form of chronic liver disease in adults, and that it may affect 10% to 35% of the worldwide population (Bellentani and Marino 2009). There is also increasing concern of NAFLD as a significant form of liver disease in pediatric populations (Berardis and Sokal, 2014; Iranikhah et al., 2018).

Described for the first time in 1988, metabolic syndrome is a condition in which many metabolic diseases or disorders [e.g., insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease (dyslipidemia), metabolic fatty liver disease, and obesity] along with vascular disorders including hypertension, thrombosis, and inflammation may be present in the same patient (Grundy 2008; Reaven 1988; Weiss et al. 2004). For example, the risk of atherosclerotic cardiovascular disease is doubled in individuals with metabolic syndrome (Grundy, 2008). Metabolic syndrome has its roots in obesity related to a sedentary lifestyle, where susceptibility factors such as adipose tissue disorders and genetic factors are also present as well. In 2016, the prevalence of obesity in the US was 39.8% in adults and 18.5% in youth (Hales et al., 2017).

Feeding SPI diet reduced liver steatosis in male obese Zucker rats compared to those fed a casein (CAS)-based diet (Hakkak et al., 2015). The exact mechanism responsible for the amelioration of liver steatosis by dietary SPI is not fully established. The SPI diet specifically targeted and halted the development of liver steatosis in this obese rat model. Microscopy analysis of liver tissue clearly showed less liver steatosis in obese rats fed the SPI-based diet compared to those fed the control CAS-based protein diet (Hakkak et al., 2015). Possibly, feeding the SPI-based diet may have altered expression of key genes associated with fundamentally important processes in the development of liver steatosis (e.g., lipid metabolism or inflammation) that counteracted the underlying genetic proclivity of these genetically obese rats to develop liver steatosis. Therefore, we have conducted a transcriptomic study to assess global gene expression in liver tissue obtained from CAS and SPI-fed rats to reveal potential gene expression signatures that were altered by feeding SPI to obese Zucker rats. The power of global expression analyses such as RNAseq is that it offers the capability of generating datasets that are hypothesis free that can lead researchers to discover new mechanisms free of constraints of hypotheses-driven research. The major goal of the RNAseq study is to identify new mechanisms that SPI is able to attenuate NAFLD. The first step in doing this is to validate the RNAseq dataset by comparing expression values to those obtained by RT-PCR. Genes that were selected for testing were ones that were most differentially expressed in liver obtained from SPI- and CAS-fed rats that were identified by Ingenuity Pathway Analysis software (Qiagen, CA). Future studies will utilize pathway analysis to reveal fundamental mechanisms associated with SPI-attenuation of liver steatosis.

## 1.2 Objectives

The major goal of the research conducted on this dissertation was to gain insight into mechanisms by which feeding SPI is able to attenuate liver steatosis in obese Zucker rats (Hakkak et al. 2015).

Specific objectives are:

1) To conduct global gene expression analysis (transcriptomics) on liver tissue obtained by Hakkak et al. (2015) from genetically obese Zucker rats fed a diet containing SPI versus Casein for 8 and 16 weeks of treatment.

2) To validate global expression datasets (transcriptomics) by targeted mRNA expression analysis using reverse transcriptase polymerase chain reaction RT-PCR.

3) To understand liver functions that might be enhanced or inhibited after 16 weeks of SPI treatment through upstream regulator analysis using Ingenuity Pathway Analysis software (Qiagen, CA).

4) To compare upstream regulator analysis obtained in global expression after 8 and 16 weeks of SPI treatment using Ingenuity Pathway Analysis software (Qiagen, CA).

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# CHAPTER 2.

# LITERATURE REVIEW

## 2.1 Metabolic Syndrome, Obesity, and Non-alcoholic Fatty Liver Disease

Described for the first time in 1988, metabolic syndrome is a condition in which many metabolic diseases or disorders [e.g., insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease (dyslipidemia), metabolic fatty liver disease, and obesity] along with vascular disorders including hypertension, thrombosis, and inflammation may be present in the same patient (Francisco et al., 2019; Grundy, 2008; Reaven, 1988). For example, the risk of atherosclerotic cardiovascular disease is doubled in individuals with metabolic syndrome (Whaley-Connell et al., 2011). Metabolic syndrome has its roots in obesity, which nowadays its known to be caused by insulin resistance and a wide range of  physiological imbalances in adipokines and proinflammatory molecules, with adipose tissue playing a major role as an endocrine organ (Kita et al., 2019).

An important medical condition linked to metabolic syndrome and obesity, nonalcoholic fatty liver disease (NAFLD) is characterized by mild steatosis, that might lead to non-alcoholic steatohepatitis (NASH), and finally to cirrhosis if damage continues. NAFLD can be described as abnormal accumulation of lipids within liver cells not associated with alcohol consumption. Traditionally, it has been considered that the course of NAFLD takes place in two stages. In the first stage, insulin resistance develops that is accompanied by lipid accumulation in the liver in the form of triglycerides. In the second stage, mitochondrial dysfunction with mitochondrial reactive oxygen species production promotes oxidative stress leading to inflammation and hepatic fibrosis (Berardis and Sokal, 2014; Iranikhah et al., 2018). A revision of the progression of pathogenesis has been proposed in which NAFLD can be tentatively diagnosed in patients with elevated liver enzymes and by imaging in the absence of other causes of liver disease. However, a definitive diagnosis can only be made by liver biopsy (Fang et al., 2018; Weiß et al., 2014). Estimations of the prevalence suggest that NAFLD is one of the most common form of chronic liver disease in adults in the US, Asia, Australia, and Europe. Moreover, it is estimated that affects 20–30% of the worldwide population, and that is higher in developed countries

(Buzzetti et al., 2016); although the prevalence of the disease has doubled in the US in recent years (Fang et al., 2018). The predominance of NAFLD in children and adolescents has also been increasing in the last decades, with an occurrence of twice in boys than in girls (Fang et al., 2018). According to Iranikhah et al., the prevalence varies between 3% and 80% in children with normal weight and over-weighted or obese children, respectively (Iranikhah et al., 2018). Although the exact causes are not well understood, there is consensus that it is the result of a combination of environmental, individual and genetic factors (Fang et al., 2018; Iranikhah et al., 2018). For this reason, the “two hits” hypothesis has been replaced by a “multiple-hit” hypothesis (Buzzetti et al., 2016; Fang et al., 2018). The multiple-hit hypothesis not only considers hereditary and environmental aspects, but it also acknowledges the importance of inflammatory pathways, and the gut-liver axis (GLA) dysfunction, that includes an imbalance on the gut microbiota, also known as intestinal dysbiosis, with modification of intestinal mucosa permeability to bacteria and or derive endotoxins (Buzzetti et al., 2016; Fang et al., 2018).

Components in the diet can regulate liver steatosis. A high saturated fat diet increases liver lipids and plasma insulin levels, inducing insulin resistance, and affecting mitochondrial function. Inflammatory stimuli play a role in the progression of NAFLD to NASH through the activation of nuclear receptors. Liver cells are involved in many pathways of lipid metabolism and also according to their location within the lobule (Tessari et al., 2009). Various factors that contribute to the dysregulation include both modifiable (e.g. obesity, insulin resistance) and non-modifiable risk factors (age-associated physiologic changes). Although there is no linear relationship between aging and prevalence of non-alcoholic fatty liver disease, current data strongly suggests that advanced age leads to more severe histological changes and poorer clinical outcomes. Hepatic lipid accumulation could lead to significant hepatic and systemic consequences including steatohepatitis, cirrhosis, impairment of systemic glucose metabolism and metabolic syndrome, thereby contributing to age-related diseases. Insulin, leptin and adiponectin are key regulators of the various physiologic processes that regulate hepatic lipid

metabolism. Recent advances have expanded our understanding in this field, highlighting the role of novel mediators such as Fibroblast growth factor (FGF) 21, and mitochondria derived peptides (Gong et al., 2017; Fang, 2018).

It is known that the development of NAFLD is accompanied by the expression of proinflammatory and immune response related molecules. Studies have been targeting CD47 for a possible therapeutic treatment in the suppression of tumor growth due to its ability to inhibit the phagocytosis mediated by macrophages function (Lee et al., 2014).

Another molecule well known by its presence during the progression of NAFLD, lipocalin-2 (LCN2), an adipokine expressed in neutrophils and involved in innate immunity (Alwahsh et al., 2014; Auguet et al., 2013; Friedl et al., 1999). Furthermore, the expression of LCN2 has been found linked to another molecules, Chemokine (C-X-C motif) ligand 9 (CXCL9) and 16 (CXCL16) (Semba et al., 2013; Tokunaga et al., 2018), cytokines involved in the induction of chemotaxis, multiplication of leukocytes, and recruitment of immune cells to the inflammation site (Tokunaga et al., 2018). CXCL9 is also present in patients with NAFLD and NASH, and it is currently being studied along CXCL10, CXCL11/CXCR3 as a possible target in cancer therapy (Tokunaga et al., 2018). Pharmacological inhibition of CXCL16 has been tested successfully in the reduction of macrophage infiltration in hepatic steatosis and hepatic damage, and therefore, a reduction in inflammation (Wehr et al., 2014).

### 2.2. Functional Food Components and Non-alcoholic Fatty Liver Disease

There is considerable evidence of the implication of dietary components, such as isoflavones in soybeans and resveratrol in wine, that can alleviate the symptoms of metabolic conditions such as liver steatosis; e.g. (Chen et al., 2015). Examples of these include isoflavones, such as genistein and daidzein, and resveratrol found in red wine.

#### 2.2.1 Genistein

Genistein is one of the most abundant isoflavones in soybean. It has been found that at the cellular level genistein inhibits cellular cholesterol synthesis and cholesterol esterification in

HepG2 human hepatoma cells (Borradaile et al, 2002). Genistein also affects fatty acid oxidation. It exerts antidiabetic and hypolipidemic effects through the upregulation of the PPAR-regulated (peroxisome proliferator-activated receptor) gene expression. Thus, the effects of genistein on cholesterol synthesis and fatty acid oxidation are well known. However, the effect of genistein on fatty acid synthesis has not yet been identified (Shin et al., 2007). Shin et al, 2007 studied the effect of genistein on fatty acid synthase (FAS) expression. FAS is not a single protein but an enzymatic system that catalyzes fatty acid synthesis. In humans, FAS is encoded by the *fasn* gene. FAS plays a central role in de novo fatty acid synthesis and in the long-term regulation of lipogenesis (Semenkovich, 1997). In the study mentioned above, the researchers reported that genistein inhibited S1P expression, which resulted in an inhibition of the SREBP-1 activation process and consequent downregulation of SREBP-1 regulated genes, such as FAS, SCD1, ACC, and GPAT in HepG2 cells (Shin et al., 2007). Genistein also improves blood pressure and restores renal function as shown in a high-fructose fed rat model (Palanisamy and Venkataraman, 2013). These beneficial effects are probably exerted by inhibitory effect on angiotensin-converting enzyme (ACE) and protein kinase C-bII (PKC-bII) activation. Genistein has also been reported to enhance adipogenesis through modification of peroxisome proliferator-activated receptor-γ (PPARγ) (Relic et al., 2009) and canonical Wnt/β-catenin signaling (Su and Simmen, 2009). Wnt signaling cascade is an important autocrine and paracrine regulator of adipogenic programming. In addition, the canonical Wnt/β-catenin pathway inhibits PPARγ and C/EBPα through downstream activation of T cell factor/lymphoid enhancer factor (TCF/LEF) (Prestwich and MacDougald, 2007). The mechanistic of such regulation remains unclear (Cain et al., 2011).

2.2.2 Daidzein

Daidzein is the second most common isoflavone in soybean. It has been related to genistein in many of its properties. A study conducted in obese diabetic Zucker rats in 2003 showed a how daidzein along with genistein could be exerting an antidiabetic role by activating

peroxisome-proliferator activator receptors (PPAR) and regulating glucose and lipid metabolism (Mezei et al., 2003). Daidzein has also been found to inhibit inducible nitric oxide synthase in activated macrophages, which in turn leads to inhibition of nitric oxide production and therefore has anti-inflammatory effects (Hämäläinen et al., 2007). It also inhibits the activation of nuclear factor-κB (NF-κB), a transcription factor of inducible nitric oxide synthase, enhancing its anti-inflammatory effect (Hämäläinen et al., 2007). Daidzein has also be found, along with genistein, to promote apoptosis in cancer colon cells by down-regulating lipid metabolism associated genes and reducing the accumulation of lipid droplets in these cells (Liang et al., 2018).

### 2.2.3 Resveratrol

Resveratrol is a natural polyphenol found in grapes, peanuts, berries, and red wine. Currently, resveratrol is used as a dietary supplement. The acceptable daily intake is 450 mg/day. In vitro, Renes et al. found that, when compared with calorie restriction, resveratrol treatment was more beneficial in reducing obesity-related metabolic complications and alleviating the inflammatory phenotype (Renes et al., 2014). Resveratrol can regulate liver lipid metabolism to prevent the development of NAFLD in animals (Mukherjee et al., 2010). Some studies conducted in rodent models have shown that resveratrol can inhibit the development of NAFLD by decreasing the levels of AST, ALT and Apo-B, as well as by decreasing body weight, blood glucose, triglycerides, total cholesterol, and LDL-cholesterol (Gómez-Zorita et al., 2012; Shang et al., 2008). In human studies, resveratrol induced SIRT1 expression and improved the human adipocyte secretome in a manner similar to that of low-glucose calorie restriction. Timmers et al. observed modest improvements in insulin sensitivity, blood pressure, metabolic rate, hepatic steatosis, and pertinent biomarkers in healthy obese males after 150 mg/day resveratrol supplementation (Timmers et al., 2011). Clearly, resveratrol has a variety of biochemical and physiological effects including the following: anti-oxidative, decreased fatty acid availability, anti-inflammatory, anti-obesity, improved lipid metabolism, and improved insulin

sensitivity (Liu et al., 2014). However, the true efficacy and mechanisms of action of resveratrol in NAFLD are not yet fully understood.

### 2.3 Effect of Feeding Soy Protein Isolate (SPI)

In 2015, Hakkak et al., compared the effects of short and long term (8 and 16 weeks respectively) of a SPI-based diet in obese Zucker rats. They were able to demonstrate that SPI exerts an anti-steatotic effect on the livers of those rats fed exclusively with SPI, and the longer the treatment the higher the benefits. SPI attenuated the liver steatosis in the Zucker rats compared with the controls. Hakkak et al., also proved that SPI reduces the levels of markers of liver damage (elevated ALT levels), and proinflammatory cytokines such as TNF and IL-6 (Hakkak et al., 2015).

It is apparent that liver steatosis was dramatically reduced in liver of obese Zucker rats fed a diet containing SPI compared to ones receiving a conventional chow. However, the reason for SPI ability to reduce liver steatosis is not apparent. By learning what effect SPI feeding had on gene expression in this obese rat model, it may be possible to develop new methods or treatments that reduce fatty liver disease and halt its progression to irreparable damage seen in cirrhosis. To this end global expression analysis studies were conducted with results presented in subsequent chapters (see Chapter 3, 4 and 5). In these studies, several genes and molecules were revealed to be potentially involved in the mitigation of NAFD. These genes are discussed below.

### 2.4 Genes-Molecules of Interest in This Dissertation

In the course of investigating gene expression in studies presented in subsequent chapters in this dissertation, a number of molecules or genes became ones of interest as they were revealed to be most differentially expressed or were predicted to be activated or inhibited in liver of obese rats fed a SPI compared to CAS-based diet. These genes are discussed briefly below.

### 2.4.1 Neuropentraxin 2 (NPTX2)

NPTX2 belongs to the pentraxin family is a member of an ancient superfamily of genes that is phylogenetically highly conserved across the animal kingdom (Mantovani et al., 2008). This superfamily can be divided according to their length and mechanisms of action into short and long pentraxins. Long pentraxins, are cytokine-inducible genes or molecules expressed in different tissues, including adipocytes, monocytes, and endothelial cells. NPTX2 is a type of neuronal long pentraxin involved in excitatory synapse formation. Moreover, NPTX2 is known to be up-regulated in Parkinson's disease and in pancreatic cancer (Park et al., 2007). The proteins of the pentraxin family are in a class of pattern recognition receptors (PRRs) and therefore are involved in acute immunological responses (Gewurz et al., 1995). Pentraxins are acute phase catalysts interacting with cytokines to modulate inflammation at both tissue and systemic levels. The plasma concentration of the short pentraxin C-reactive protein, secreted by the liver, is an approved marker as a diagnostic tool of systemic inflammation in obese individuals (Barazzoni et al. 2016; Mantovani et al. 2008). However, pentraxin 3 (PTX3), which is a typical structure for long pentraxins, has been reported to limit tissue damage and the inflammatory process in several disease models, including atherosclerosis, myocardial infarction, kidney injury, and experimental carcinogenesis (Barazzoni et al., 2016; Mantovani et al., 2008). As NAFLD and obesity are associated with a general systemic inflammation due to elevated circulating levels of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL) 6, the increase in NPTX2 expression in SPI-fed rats might be an intrinsic mechanism that functions to attenuate systemic inflammation in NAFLD.

In addition, there are epigenetic studies focused on aging that relate several components found in the diet, such as epigallocatechin-3-gallatte (EGCG) from green tea and genistein from soybeans, that could be controlling gene expression through DNA methylation (Johnson et al., 2012). This type of DNA methylation can occur not only on the coding regions of specific genes of interest, but also on their promoters (Bacalini et al., 2014). In these studies,

the gene NPTX2 is indicated as one of the most important genes under epigenetic influence (Bocklandt et al., 2011), along with tumor suppressor genes such as hMLH1 and RARb (Bacalini et al. 2014; Johnson et al., 2012).

2.4.2. Interleukin 33 (IL33)

IL33 belongs to the IL1 superfamily of cytokines expressed in healthy vascular endothelial cells, epithelial cells, fibroblasts and smooth muscle cells (Haraldsen et al., 2009; Miller, 2011). IL33 is expressed primarily in cells from tissues involved in the production and maintenance of a barrier. When the barriers are damaged, IL33 behaves as an alarm, being released and initiating the acute local inflammation and tissue-repair process. IL33 has a function as a ligand for the Th2-associated ST2 receptor activating NFkB and mitogen-activated kinases. IL33 also has a function as a nuclear factor; thus, regulating gene transcription (Cayrol and Girard, 2014; Haraldsen et al., 2009; Martin and Martin, 2016; Miller, 2011). IL33 was reported to be a regulator of hepatic ischemia and reperfusion injury related with the activation of NFκB, p38 MAPK, cyclin D1, and Bcl-2 that restricts liver injury and decreases inflammation promoters in mice (Sakai et al., 2012). In a recent review, Sun et al., reported that IL33 may serve a protective role in fatty liver disease (Sun et al., 2017). Pejnovic et al., developed an NAFLD mouse model through feeding a high fat diet. Treating these NAFLD mice fed a high fat diet with IL-33 ameliorating hepatic steatosis as well as insulin resistance and glucose intolerance (Pejnovic et al., 2016). Furthermore, treating genetically obese mice with IL33 reduced fat accumulation (adiposity), possibly through induction of Th2-mediated cytokine production (Miller et al., 2010). Thus, it is possible that the elevation of IL33 in SPI-fed rats played a role in the reduction of liver steatosis compared to CAS-fed rats in the present study. There are some studies relating consumption or treatment with phytoestrogens with immune response, mostly when members of IL1 superfamily are involved, and chronic diseases and other conditions, such as asthma, inflammatory bowel diseases, and radiation-induced bone marrow failure (Ha et al., 2013; Martin and Bolling, 2015; Sandoval et al., 2010; Tanaka and

Takahashi, 2013). Nevertheless, little is known regarding the effects of IL33 in the presence of phytoestrogens or any isoflavones in NAFLD.

2.4.3 Serine Protease

2.4.3a Protease, serine 32 (PRSS32).

The PRSS32 gene is located in chromosome 10 of *Rattus norvegicus* and has a biased expression in liver and uterus (https://www.ncbi.nlm.nih.gov/gene/30297). Tryptase-5 protein in mouse is encoded by *Prss32* gene, which is located on chromosome 17, and corresponds to a functional trypsin-like serine protease (Wong et al., 2004).

2.4.3b Protease, serine 8 (PRSS8).

PRSS8, also known as prostasin and channel-activating protease 1 (CAP1), is a trypsin-like serine peptidase. PRSS8 was first identified as a secreted prostate gland product (Tong et al., 2004; Yan et al., 2014). In humans, PRSS8 mRNA expression has been detected in prostate, liver, salivary gland, kidney, lung, pancreas, colon, bronchus, and in some cells from the kidney (Chen and Chai, 2012; Yu et al., 2014). Uchimura et al., 2014 reported that upregulation of PRSS8 protected mice from chronic inflammation by reducing toll-like receptor 4 (TLR4) attachment to the endoplasmic reticulum (Uchimura et al., 2014). Furthermore, down-regulation of PRSS8 in mice fed a high fat diet contributed to hepatic insulin resistance and development of diabetes.

2.4.4 Cytochrome P450 genes

The cytochrome P450 superfamily (CYP) is widely present and prominent in both prokaryotes and eukaryotes. CYP superfamily is classified in families and subfamilies based on amino acid sequence affinity (Honkakoski and Negishi, 2000; Xu et al., 2005). Numerous members of this superfamily are involved in electron transport chain systems, having its name derived from its absorption peak at 450 nm in the visible spectrum/spectra (Danielson, 2005). The proteins of this superfamily contain a heme group to oxidize their substrates and function as mono-oxygenases, playing a major protective role from toxic and oxidative damage in the liver,

intestine, kidney, and lung (Gonzalez and Gelboin, 1992; Xu et al., 2005). CYPs act in a vast range of functions from the synthesis and metabolism of fatty acids, steroid hormones, cholesterol, bile acids, and vitamins to drug metabolism and detoxification from xenobiotics and therefore, have an important role in both exogenous and endogenous substrate metabolism (Honkakoski and Negishi, 2000; Xu et al., 2005). In humans, the importance of studying and understanding CYPs mechanisms lies in drug metabolism and interactions (Danielson, 2005). Many members of the CYP superfamily are induced in liver after exposure with xenobiotics (Pavek and Dvorak, 2008), including those present in the diet. Families CYP1, CYP2 and CYP3, to which our analyzed genes belong, are inside this group induced by xenobiotics (Pavek and Dvorak, 2008). There is a great abundance of studies involving the interaction between CYPs and specific dietary components (Hamilton-Reeves et al., 2007; Kishida et al., 2004; Li et al., 2007; Liu et al., 2016; Ronis, 2016). Current studies indicate that consumption of SPI protects against cancer by down-regulating the expression of CYP1 family members (Rowlands et al., 2001). There are also several reports where the expression of CYP3 and CYP4 families was activated or up-regulated by a SPI-diet (Badger et al., 2008; Li et al., 2009; Mezei et al., 2003; Ronis et al., 2004). In a recent study, it was demonstrated that although SPI maternal consumption mitigates serum lipid levels on rat male offspring mainly through the up-regulation of important CYPs such as CYP3A1, the liver protein CYP2C12 was down-regulated in the SPI-fed group compared to the CAS-fed group (Won et al., 2017). This finding coincides with our results in this study.

2.4.5 Sulfotransferase family 2A Member 1 (SULT2A1)

SULT2A1 is a member of the sulfotransferase family, whose members are divided into families and subfamilies based on their amino acid sequence (Huang et al., 2010). Sulfotransferases assist in the metabolism of endogenous compounds and drugs through the biochemical process of sulfation, transforming these substances into more hydrophilic water-soluble molecules that can be easily eliminated from the organism. These proteins catalyze the

sulfation of steroids, bile acids, neurotransmitters and thyroid hormones in the liver and adrenal glands (Yalcin et al., 2013). There are cell membrane bound sulfotransferases and cytosolic sulfotransferases; SULT2A1 is a cytosolic sulfotransferase (Huang et al., 2010; Nowell and Falany, 2006). Protective effects of diet, mostly soy derived products, against cancer have been reported through sulfation of flavonoids and other phenolic compounds by cytosolic sulfotransferases (Pai et al., 2001). SULT1 and SULT2 enzymes showed N-sulfating activities for carcinogenic heterocyclic amines. SULT2A1 has been shown to sulfate and thus further bioactivate the classical hepatic procarcinogen N-hydroxy-2-acetylamino-fluorone (Pai et al., 2001). The detoxification function of liver is illustrated by SULT2A1, playing a major role in bile acid homeostasis and protection against their toxic effects. SULT2A1 is also found at minor levels in jejunum, ileum, cecum and kidney cytosol samples (Prima et al., 2013). Mesia-Vela and Kauffman (2003) determined that when flavonoids are present in the diet they become potent inhibitors of sulfotransferases such as SULT1A1 in the liver (Mesía-Vela and Kauffman, 2003). This study also stated that the potency of the inhibition of SULT1A1 by flavonoids could be related to the number and position of hydroxyl groups present in the molecules of such proteins, with genistein presenting a higher level of inhibition than daidzein. Nevertheless, Mesia-Vela and Kauffman (2003) did not find a correspondence between number and position of hydroxyl groups present in flavonoids to inhibit the sulfotransferase SULT2A1, and the inhibition is less potent that the one exerted over SULT1A1. Yalcin et al. (2013) reported that there was a clear decrease in several sulfotransferases in a progression from a healthy normal liver, to steatosis, to cirrhotic livers resulting from diabetes and alcohol (Yalcin et al., 2013).

### 2.4.6 Regucalcin (RGN)

RGN is a unique calcium-binding protein that does not contain the EF-hand motif[1] of calcium-binding domain (Yamaguchi and Murata, 2013). RGN is an androgen-independent factor that

[1] The EF-hand motif is the most common calcium binding motif in proteins (Lewit-Bentley and Rety, 2000. EF-hand calcium-binding proteins. Curr Opin Struct Biol, 2000 10:637-43)

decreases with aging (Ishigami et al., 2004). Since the location of RGN is the nucleus, it is believed to be implicated in the regulation of gene expression. RGN was originally discovered in 1978 as unique calcium-binding protein that does not contain the EF-hand motif of calcium-binding domain. The gene (*rgn*) is localized on the X chromosome and is identified in over 15 species. It plays a multifunctional role in cell regulation, maintaining of intracellular calcium homeostasis and suppressing of signal transduction, translational protein synthesis, nuclear deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, proliferation, and apoptosis in many cell types. RGN may play a pathophysiological role in metabolic disorder. The expression of RGN is stimulated through the action of insulin in liver cells *in vitro* and *in vivo* and it is decreased in the liver of rats with type I diabetes induced by streptozotocin administration *in vivo*. Overexpression of endogenous RGN stimulates glucose utilization and lipid production in liver cells with glucose supplementation in vitro. RGN can reveal insulin resistance in liver cells (Yamaguchi and Murata, 2013). Deficiency of RGN induces an impairment of glucose tolerance and lipid accumulation in the liver of mice *in vivo*. Ishigami et al. (2004) demonstrated that hepatocytes from RGN-deficient mice revealed many lipid droplets, and abnormally enlarged mitochondria and lysosomes (Ishigami et al., 2004). RGN deficiency in mice has a profound effect on the metabolism of neutral lipids and phospholipids such as phosphatidylethanolamine, cardiolipin, phosphatidylcholine, phosphatidylserine, and sphingomyelin. Thus, it is likely that this anomaly in the lipid metabolism might decrease the life span of RGN-deficient mice (Ishigami et al., 2004). The overexpression of endogenous RGN has been shown to decrease triglyceride, total cholesterol, and glycogen contents in the liver of rats, inducing hyperlipidemia. Leptin and adiponectin mRNA expressions in the liver tissues are decreased in RGN transgenic rats. Decrease in hepatic RGN is usually associated with the development and progression of NAFLD and fibrosis in human patients. RGN may be a key molecule in lipid metabolic disorder and type II diabetes (Yamaguchi and Murata, 2013). According to a database search, RGN is a

unique protein, wholly separate from any other family of proteins and has a domain that resembles that of bacterial and yeast RNA polymerase (Ishigami et al., 2004).

2.4.7 Regulated Endocrine Specific Protein 18 (Resp18)

Resp18 expression is limited to neuroendocrine tissues and sperm (Bloomquist et al., 1994; Schiller and Darlington, 1996). However, very little is known about this gene or the protein that it encodes (Zhang et al., 2007) and even less about its relationship with SPI and NAFLD. Resp18 shares sequence homology with the luminal region of IA-2, a dense core vesicle (DCV) transmembrane protein involved in insulin secretion -involved in type I diabetes (Zhang et al., 2007). Although the human version of this protein is not expressed in liver, according to the webpage for Resp18 gene on NCBI (Provisional) (https://www.ncbi.nlm.nih.gov/gene/50561) this gene is expressed in lower concentrations in rat liver, suggesting that it may not be a functional protein. Zhang et al. (2007) cloned two Resp18 splice variants. Resp18-alpha encodes a deduced 173-amino acid protein, and Resp18-beta encodes a deduced 228-amino acid protein (Zhang et al., 2007). Both proteins share significant similarity with IA2. Northern blot analysis of several human tissues detected strong expression of a 0.8-kb transcript in pancreas and weak expression of 0.8- and 1.2-kb transcripts in placenta. Western blot analysis detected Resp18 in mouse pancreas, in mouse and rat insulin-secreting beta cells, and in mouse pituitary corticotropic cells, but not in mouse fibroblasts or human colon carcinoma or HeLa cells (Zhang et al., 2007). Immunofluorescence analysis of rat pancreas detected Resp18 in most secretory islet cells examined, and a similar distribution was found in human pancreas. Immunoelectron microscopy of rat islets localized Resp18 to dense-core vesicles, endoplasmic reticulum, and Golgi. Zhang et al. (2007) identified Resp18 orthologs only in mammalian genomes (Zhang et al., 2007).

2.4.8 Cell death induced DFFA like effector A (Cidea)

Cidea is a member of the CIDE family protein, as well as Cideb and Cidec, and are mitochondrial lipid-droplet-associated proteins involved in regulation of lipid storage and the

formation of large LDs in adipocytes and hepatocytes (Tiniakos et al., 2010; Zhou et al., 2012; Carr and Ahima, 2016). Cidea can induce apoptosis through a caspase-independent pathway (Zhou et al., 2003; Tiniakos et al., 2010). Cidea is well known to be expressed in white and brown adipose tissue, and in liver. Moreover, Cidea has been directly related with the development of NAFLD (Zhou et al., 2003; Tiniakos et al., 2010; Wu et al., 2014). In addition, knockout mice for Cidea and leptin-deficient mice have shown to be lean and obesity resistant (Zhou et al., 2003; Tiniakos et al., 2010; Wu et al., 2014). Cidea is also up-reglated in the presence of insulin (Wang et al., 2010).

2.4.9 Guanine nucleotide-binding protein G(i), α-1 subunit (Gnai1),

Gnai1 encodes the alfa subunit of a Guanine nucleotide-binding protein (G protein). G proteins are important signal transducers present in all eukaryotes, usually located in the cell membrane, that communicate signals from many hormones, neurotransmitters, chemokines, and autocrine and paracrine factors (Neves et al., 2002; Jones and Assmann, 2004). Moreover, G proteins participate in intricate pathways from cell surface receptors with many effectors downstream. Almost all G protein pathways either stimulate or inhibit one or more of the Mitogen-Activated Protein Kinase (MAPK) signaling pathways (Neves et al., 2002). All G proteins are composed of three subunits, alpha, beta and gamma. In general, G proteins behave as dimers when signaling, although they are heterotrimers, communicating either through G alpha subunit or G beta-gamma complex (Neves et al., 2002). However, in the Gi pathway both portions of the G protein, G-alpha subunit and G-beta-gamma complex, can transmit signals (Neves et al., 2002). Many G proteins have been found by biochemical purification after the first four were described (Gs, Gt, Gi, and Go) and their subunits identified by cDNA cloning (Neves et al., 2002; Simon et al.,1991). Gnai1 can regulate cell proliferation and differentiation, assist platelet aggregation, and act as receptors in multiple cancers (Nguyen et al., 2018). Acording to Yao et al., (2012), Gnai1 is significantly down-regulated in hepatocellular carcinoma (HCC) compared with normal liver (Yao et al., 2012). Gnai1 is

hypothesized to function as an inhibitor of HCC migration and invasion (cancer invasion), and it has been hypothesized by Nguyen et al., 2018, that Gnai1 is part of a cell mechanism to impede tumor growth (Nguyen et al., 2018).

2.4.10. Human immunodeficiency virus type 1 enhancer binding protein 2 (HIVEP2)

HIVEP2 is a zinc finger transcription factor located on the chromosome 1 of *Rattus norvegicus* genome, and on chromosome 6 in *Homo sapiens* (Fujii et al., 2005). HIVEP2 contains a ZAS domain composed of two zinc finger motifs, a stretch of highly acidic amino acids and a serine/threonine-rich sequence. This protein binds a specific DNA sequence similar to the κB motif present in the Nuclear Factor-κB, usually found in regulatory regions of different cellular and viral genes that might be involved in growth, development and metastasis, including those of SV40, CMV, or HIV1. Moreover, related sequences are also found in the enhancer elements of various cellular promoters, such as those of the class I MHC, IL2 receptor, somatostatin receptor II, and interferon-beta genes (Wu, 2002). HIVEP2 gene role has been related with the regulation of immune responses and cellular proliferation (Fujii et al., 2005). HIVEP2 mRNA is mostly detected in the brain, heart and immune cells (Campbell and Levitt, 2003; Makino et al., 1994; Ron et al., 1991). HIVEP2 deficient mice have a defect in T cell development, have reduced white adipose tissue, a deficiency associated to defective Bone Morphogenetic Protein 2 (BMP) dependent adipogenesis and are also hypersensitive to stress (Jin et al., 2006; Takagi et al., 2006). HIVEP2 translocates into the nucleus as a consequence of BMP-2 stimulation (Shukla and Yuspa, 2010). HIVEP2 is considered to have a key role in the activation and function of NK cells and the development of T cell lymphoma (Yamashita et al., 2012). Vertebrates have at least three orthologs of HIVEP2, namely HIVEP-1, HIVEP-2 and HIVEP-3 (Wu, 2002). HIVEP genes encode transcriptional proteins that activate or repress the transcription of a variety of genes involved in growth, development, and metastasis (Wu, 2002). Allelic loss on human chromosomal locus of HIVEP3, has been usually reported in a variety of tumors, including breast cancer, liver cancer, and B cell lymphoma. Moreover, changes in gene

expression of HIVEP1 and HIVEP2 have been associated with poor prognosis in chronic lymphocytic leukemia patients. Thus, appropriate expression of the HIVEP proteins might be required to maintain normal growth (Wu, 2002).

2.4.11 Melanoma-associated antigen E1 (MAGEE1).

The first member of the MAGE superfamily has been described in relation to melanoma cancer cells in 1991 (Chomez et al. 2001). MAGEE1 is a member of a superfamily of genes that in humans spans about 60 different types of proteins, and most of their genes are located in a cluster on the chromosome X (Katsura and Satta 2011). The superfamily, whose members all encode a MAGE homology domain, is divided in two types, type I and type II. Type I MAGE family includes MAGE-A, -B, and C; while type II contains MAGE-D, -E, -F, -G, -H, and -L members (Lian et al. 2018). The expression of MAGE superfamily members is highly involved in cancer development and progression as well as normal functions in embryonic structures and somatic and stem cells differentiation (Gordeeva et al. 2019). However, their functions remain unknown.

2.4.12. Short-chain dehydrogenase/reductase family 16C member 6 (Sdr16c6)

Sdr16c6 belongs to the short-chain dehydrogenases/reductases (SDR) superfamily, an ancient superfamily of enzymes spanning all domains of life, whose members consist of NAD(P)(H)-dependent oxidoreductases (Kallberg et al., 2010; Kavanagh et al., 2008). Although its members share sequence motifs and similar mechanisms, sequence identities are low (Kallberg et al., 2010; Kavanagh et al., 2008). The human ortholog of Sdr16c6 is a pseudogene (Adams et al., 2017; Kedishvili, 2016). SDRs have a role in the metabolism of lipids, hormones, prostaglandins, carbohydrates, amino acids, retinoic acid, and xenobiotics, among others. They also participate in redox sensor systems (Kavanagh et al., 2008; Persson et al., 2009). All SDRs have in common a Rossmann-fold domain[2] and their capacity to bind NAD(P) dinucleotides

[2] From https://proteopedia.org/wiki/index.php/Rossmann_fold. The Rossmann fold is a super-secondary structure characterized by an alternating motif of beta-strand-alpha helix-beta strand secondary structures called a βαβ

(Kavanagh et al., 2008; Persson and Kallberg, 2013). The retinol dehydrogenase Rdhe2, homologous to *Sdr16c5* and *Sdr16c6*, has an important role in frog embryonic development (Belyaeva et al., 2012). In the annotated genome of the amphibian *Xenopus tropicalis* there is one gene (*rdhe2*) homologous to the human version of *Sdr16c5* and *Sdr16c6*, which encode Retinol Dehydrogenase Epidermal 2 (RDHE2 or Sdr16c5) and Retinol Dehydrogenase Epidermal 2-Similar (RDHE2S or Sdr16c6) respectively. These genes appeared to have been recently originated by a duplication event (Belyaeva et al., 2015). According to Belyaeva et al. (2012) the product of *Sdr16c6* gene still has not been characterized in any species (Belyaeva et al., 2012). However, it seems logical to expect it could be displaying a similar function than Sdr16c5 in the metabolism of retinoic acid (Belyaeva et al., 2012). Sdr16c6 is one of the genes affected by vitamin D supplementation in an animal model for Intrahepatic cholangiocarcinoma (ICC), an aggressive cancer (Chiang et al., 2014). It has a primarily found in liver, lung and muscle in rat and located in chromosome 5 (https://www.ncbi.nlm.nih.gov/gene/502939#gene-expression). In *Rattus norvegicus*, it is predicted to localize to the lipid droplet (https://rgd.mcw.edu/rgdweb /report/gene/main.html?id=1562060). According to NCBI, is highly expressed in rat liver at 21 weeks of age (https://www.ncbi.nlm.nih.gov/gene/502939).

2.4.12 Histidine Ammonia Lyase (histidase – HAL).

HAL catalyzes the first reaction in the histidine degradation pathway. HAL is a cytoplasmic enzyme present in liver and skin tissue and catalyzes the oxidative deamination of L-histidine to produce urocanic acid and ammonia (Tovar et al., 2002). At the transcriptional level, HAL is regulated in the liver by the protein content in the diet. The amounts of most amino acid-degrading enzymes in the liver increase as protein intake rises and decrease as protein intake falls (Torres et al., 1998). When the histidine requirement has been met, increased HAL

fold. The beta strands participate in the formation of a beta-sheet. The βαβ fold structure is commonly observed in enzymes that have dinucleotide coenzymes such as FAD, NAD, and NADP.

expression and activity enables the liver to catabolize and eliminate excess histidine after the ingestion of a high protein diet. Thus, regulating the histidine concentration in plasma by controlling the food consumption of histidine is an important role that HAL plays at the cellular and organismal level (Torres et al., 1998; Tovar et al., 2002). It was determined that both casein and soy increase hepatic HAL levels (Tovar et al., 2002). However, the groups of rats with higher intake of both proteins (casein or soy at 50% versus 18%) triggered higher levels of HAL than the groups with lower intake of the same two protein sources (Tovar et al., 2002). Gene expression of HAL has been reported to be regulated by estrogen, glucagon, and glucocorticoids generated during a catabolic state (Armstrong and Feigelsong, 1980; Tovar et al., 2002). It has been demonstrated that the administration of glucagon to rats promoted the expression of HAL (Alemán et al., 1998).

2.4.13 Glutamate pyruvate transaminase (GPT)

GPT, also known as alanine aminotransferase, plays a key role in the intermediary metabolism of glucose and amino acids. Specifically, it is an enzyme that catalyzes the reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate. Thus, GPT plays an important role in gluconeogenesis and amino acid metabolism (Jadaho et al., 2004). Serum activity levels of this enzyme are routinely used as a biomarker of liver injury caused by drug toxicity, infection, alcohol, and steatosis. Although elevated GPT is considered a biomarker of NAFLD, caused in part by insulin resistance characteristic of metabolic syndrome (Calcaterra et al., 2011), this hypothesis has been challenged due to the difficulty in establishing a direct correlation between the circulating GPT levels and the exact stage of the NAFLD. Despite the fact that GPT exists as two isoforms (GPT1 and GPT2, respectively) coded by two different genes located on different chromosomes in humans, the enzymatic activity in circulation is measured as total GPT (Sookoian et al., 2016). It is also known that both isoforms not only have different expression pattern in other tissues, but also different intracellular localizations. According to a recent study (Sookoian et al., 2016), since GPT2 is the

mitochondrial isoform of this enzyme, circulating levels of GPT should be considered as sensors of global metabolic deregulation, including mitochondrial energetic control, rather than as merely a biomarker.

2.4.14 Indolethylamine N-methyltransferase (INMT)

INMT catalyzes the N-methylation of indoles such as tryptamine and related compounds and a major mechanism for degradation of endogenous and exogenous compounds (Thompson et al., 1999). However, the function of INMT and the physiological significance of the N-methylation pathway of indolethylamine metabolism is still not clear (Kärkkäinen et al., 2005; Thompson et al., 1999). The expression of INMT mRNA has been found in several mammalian tissues with the highest expression in the thyroid, adrenal gland, and lungs; very low or absent expression has been reported in brain, spleen, thymus, peripheral blood leukocytes, liver, and kidney (Thompson et al., 1999). It is also present in most of stromal and epithelial cells of organs related to the autonomous nervous system but absent from neurons and striated muscle cells (Kärkkäinen et al., 2005). INMT was indicated to be present in a fatty liver gene set as part of the human phenotype ontology project (Köhler et al., 2014), but what role it plays in fatty liver disease is not apparent at this time.

2.4.15 Serpin family A member 6 (SerpinA6)

SerpinA6 belongs to the broadly distributed family of protease inhibitors called serpins. Serpin-like genes have been described in numerous phyla, from viruses to animals (Law et al., 2006). Most serpins inhibit serine proteases, but there are some rare serpins performing a non-inhibitory function., SerpinA6 which encodes an alpha-globulin also called corticosteroid-binding globulin (CBG) or transcortin, is primarily produced in the liver and to a lesser extent in the placenta, kidney, endometrium, lung, pituitary, and hypothalamus (Gagliardi et al., 2010; Hammond et al., 1987; Law et al., 2006). SerpinA6 is a transporter of several anti-inflammatory steroids and progesterone in plasma (Henley and Lightman, 2011). Under normal conditions in humans, about 80% to 90% of cortisol is bound to SerpinA6 with high affinity (Gagliardi et al.,

2010; Hammond et al., 1987; Richard et al., 2010). By regulating the free cortisol concentration in blood, SerpinA 6 controls the bioavailability of corticosteroids, thus acting as a buffer during a secretory surge that can increase the levels of cortisol or as a reservoir of this corticosteroid during periods of decreased secretion (Gagliardi et al., 2010; Henley and Lightman, 2011). SerpinA6 levels in blood are associated with body mass index, insulin resistance, serum levels of interleukin-6, and adiponectin as well as proliferation and differentiation of preadipocytes (Braun et al., 2010). The role of SerpinA6 as a simple transporter is starting to change in the last few years. SerpinA 6 is the substrate of elastase that is released in high concentration by activated neutrophils at sites of inflammation. Once elastase cleaves SerpinA 6, the binding affinity for cortisol is reduced 10-fold, releasing cortisol at the inflammation site as a result. Thus, SerpinA 6 might have a key role in preventing tissue damage at inflammatory sites, such as that occurring in NAFLD, since cortisol modulates inflammatory response (Braun et al., 2010; Gagliardi et al., 2010).

2.4.16 Ajuba LIM Protein (AJUBA).

AJUBA belongs to the LIM protein family, that is characterized by tandem homologous C-terminal LIM domains and a unique N-terminal preLIM region rich in glycine and proline residues (Fan et al., 2015). This arrangement contributes to the linking and/or strengthening of epithelial cell-cell junctions in part by linking adhesive receptors to the actin cytoskeleton. The LIM motif is a double zinc finger structure and functions as a protein-protein interface (Fan et al., 2015). In humans, AJUBA expression was elevated in patients exhibiting nonalcoholic steatohepatitis (NASH) compared to patients with healthy liver, but there were no differences in expression between patients with NAFLD compared to those with healthy liver (Arendt et al., 2015).

2.4.17 Colony stimulating factor 2 receptor subunit beta (CSF2RB)

The CSF2RB protein is the common beta chain subunit of the high affinity receptor for Interleukin 3 (IL3), IL5, and granulocyte-macrophage colony-stimulating factor receptor (GM-

CSF receptor); these are all cytokines and important regulators of hematopoiesis and inflammation (Akdis et al., 2011; Woodcock et al., 1994). Tallino et al. (2015) reported that a copper deficient diet up-regulated CSF2RB in a NAFLD mature rat model (Tallino et al., 2015).

2.4.18. Tumor necrosis factor receptor superfamily member 12A (TNFRSF12A).

TNFRSF12A is a transmembrane protein that causes a weak induction of apoptosis in some cell types. The human TNFRSF12A gene is expressed at relatively low levels in normal liver tissue but at high levels in liver cancer cell lines and in hepatocellular carcinoma specimens (Feng et al., 2000) and the expression of this protein increases in human liver tissue exhibiting NAFLD (Jakubowski et al., 2005; Wiley et al., 2001). TNFRSF12A promotes angiogenesis and the proliferation of endothelial cells and modulates cellular adhesion to matrix proteins and inhibits growth and migration in vitro (Feng et al., 2000). Nuclear factor of activated T-cells 1 (NFAT1), a type of transcription factor, regulates the expression of TNFRSF12A and its ligand, tumor necrosis factor-like weak inducer of apoptosis (TWEAK), with Lipocalin 2 to increase breast cancer cell invasion (Gaudineau et al., 2012).

2.4.19 Lipocalin-2 (LCN2)

This molecule belongs to the heterogeneous lipocalin (LCN) family whose members display differences at the sequence level but remain similar at the structural level (Flower, 1996). The LCN family is involved in the transport of small hydrophobic molecules and other various functions (Borkham-Kamphorst et al., 2013). LCN2 is a secretory glycoprotein, with different functions ranging from transport of small molecules such as fatty acids, steroids, thyroid hormone, and retinoids, to a key role in the innate immunity, iron trafficking, and induction of apoptosis (Auguet et al., 2011; Flower, 1996). LCN2 is considered an adipocytokine (Alwahsh et al., 2014; Auguet et al., 2013) and reported to be secreted as a cytokine from the liver and kidney as well as from neutrophils, and macrophages (Esteve et al., 2009; Wang et al., 2007). Cell-based immunohistochemistry studies affirm that the main source of LCN2 in liver are the injured hepatocytes themselves (Borkham-Kamphorst et al., 2013). The presence of LCN2

protein in blood and urine is an early biomarker of acute kidney injury and other pathologic conditions (Asimakopoulou et al., 2014; Borkham-Kamphorst et al., 2013; Makris et al., 2012). It was reported that LCN2 is likely to be one of the adipokines implicated in the pathogenesis of NAFLD since it is secreted from both adipose tissue and the liver (Auguet et al., 2013). In a diet-inducible fatty liver rat model, the researchers found a strong correlation between a high calorie diet and an increase or up-regulation of hepatic LCN2, elevated indicators of apoptosis, mitochondrial dysfunction, and lipid peroxidation in the hepatic cells (Alwahsh et al., 2014).

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**CHAPTER 3**

Comparison of Liver Gene Expression by RNAseq and PCR Analysis After 8 Weeks of Feeding Soy Protein Isolate- or Casein-based diets in an Obese Liver Steatosis Rat Model.

**Published 2019. Food & Function. DOI:10.1039/c9fo0138.**

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Running title: Effects of soy protein on gene expression in liver steatosis model

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ABSTRACT

Previously, we reported that feeding soy protein isolate (SPI) reduced liver steatosis in obese rats compared to those fed a casein (CAS)-based diet; however, the mechanism for this protection is unknown. To gain insight into the ability of SPI to ameliorate liver steatosis, we conducted transcriptomic (RNAseq) analysis on liver samples from obese rats fed either the SPI- or CAS-based diets (n=8 per group) for 8 weeks using an Ilumina HiSeq with 100 base paired end reads for sequencing. Data were analyzed by Ingenuity Pathway Analysis (IPA) software using a $P < 0.05$ and 1.3-fold differential expression cutoff values between the SPI- and CAS-based groups. To independently validate the RNAseq data, we conducted targeted mRNA expression analysis using quantitative PCR (qPCR) on a subset of differently expressed genes. The results indicate that mRNA expression by qPCR concurred with RNAseq for NPTX2, GPT, INMT, and HAL that were up-regulated in SPI-fed rats ($P < 0.05$) and PRSS8, AJUBA, CSF2RB, and Cyp2c12 that were down-regulated ($P < 0.05$) in SPI-fed rats compared to CAS-fed rats. Our findings may shed light on understanding mechanisms enabling SPI diet to reduce liver steatosis in this obese Zucker rat model.

**Key words:** obesity, non-alcoholic fatty liver disease, soy protein, gene expression

## INTRODUCTION

Described for the first time in 1988, metabolic syndrome is a condition in which many metabolic diseases or disorders [e.g., insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease (dyslipidemia), metabolic fatty liver disease, and obesity] along with vascular disorders including hypertension, thrombosis, and inflammation may be present in the same patient (Grundy 2008; Reaven 1988; Weiss et al. 2004). For example, the risk of atherosclerotic cardiovascular disease is doubled in individuals with metabolic syndrome (Grundy 2008). Metabolic syndrome has its roots in obesity related to a sedentary lifestyle, where susceptibility factors such as adipose tissue disorders and genetic factors are also present as well. In 2016, the prevalence of obesity in the US was 39.8% in adults and 18.5% in youth (Hales et al. 2017).

An important medical condition linked to metabolic syndrome and obesity, nonalcoholic fatty liver disease (NAFLD) is characterized by mild steatosis, that if left unchecked, leads to non-alcoholic steatohepatitis (NASH), and finally to cirrhosis if damage continues. NAFLD can be described as abnormal accumulation of lipids within liver cells not associated with alcohol consumption. The course of NAFLD takes place in two stages. In the first stage, insulin resistance develops that is accompanied by lipid accumulation in the liver in the form of triglycerides. In the second stage, mitochondrial dysfunction with mitochondrial reactive oxygen species production promotes oxidative stress leading to inflammation and hepatic fibrosis (Day and James, 1998; Masterton et al., 2010). Recently, a revision of the progression of pathogenesis has been proposed in which NAFLD can be tentatively diagnosed in patients with elevated liver enzymes and by imaging in the absence of other causes of liver disease. However, a definitive diagnosis can only be made by liver biopsy (Serviddio et al., 2008; Weiß et al., 2014). Estimations of the prevalence suggest that NAFLD could be the most common form of chronic liver disease in adults in the US, Asia, Australia, and Europe that may affect 10% to 35% of the worldwide population (Bellentani and Marino 2009). There is also increasing concern

of NAFLD as a significant form of liver disease in pediatric populations (Cornier et al. 2008; Kleiner et al. 2005).

Feeding SPI diet reduced liver steatosis in male obese Zucker rats compared to those fed a casein (CAS)-based diet (Hakkak et al. 2015). The exact mechanism responsible for the amelioration of liver steatosis by dietary SPI is not fully established. The SPI diet specifically targeted and halted the development of liver steatosis in this obese rat model. Microscopy analysis of liver tissue clearly showed less liver steatosis in obese rats fed the SPI-based diet compared to those fed the control CAS-based protein diet (Figure 1) (Hakkak et al. 2015). Possibly, feeding the SPI-based diet may have altered expression of key genes associated with fundamentally important processes in the development of liver steatosis (e.g., lipid metabolism or inflammation) that counteracted the underlying genetic proclivity of these genetically obese rats to develop liver steatosis. Therefore, we have conducted a transcriptomic study to assess global gene expression in liver tissue obtained from CAS and SPI-fed rats to reveal potential gene expression signatures that were altered by feeding SPI to obese Zucker rats. The power of global expression analyses such as RNAseq is that it offers the capability of generating datasets that are hypothesis free that can lead researchers to discover new mechanisms free of constraints of hypotheses-driven research. The major goal of the RNAseq study is to identify new mechanisms that SPI is able to attenuate NAFLD. The first step in doing this is to validate the RNAseq dataset by comparing expression values to those obtained by RT-PCR. Genes that were selected for testing were ones that were most differentially expressed in liver obtained from SPI- and CAS-fed rats that were identified by Ingenuity Pathway Analysis software (Qiagen, CA). Future studies will utilize pathway analysis to reveal fundamental mechanisms associated with SPI-attenuation of liver steatosis.

## MATERIALS AND METHODS

### Animal Procedures and Diets

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences (Protocol code number 3242; approved on 12/6/2011). Liver tissue was obtained from male obese Zucker rats from a previous study (Hakkak et al. 2015). Briefly, rats (6 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). After one week of acclimation, rats were randomly assigned to either a casein (CAS) diet or soy protein isolate (SPI) diet. They were housed in individual cages and provided the diets ad libitum for 8 weeks. At 15 weeks, the rats were humanely killed, and liver samples obtained, and flash frozen in liquid nitrogen and stored at −80°C.

### Transcriptomic Analysis

RNA was extracted from liver samples using phenol chloroform. One percent agarose gel electrophoresis was used to evaluate the quality of RNA and concentrations were assessed with Take 3 micro volume plate utilizing Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). The RNA samples were sent for sequencing to the Research Support Facility at Michigan State University (East Lansing, MI). Illumina HiSeq 100 base pair paired end read was used for global expression analysis by RNA sequencing (RNAseq). To map the reads to *Rattus rattus* genome assembly version 4, we used CLC Genomics Workbench 8 software that adopts the pipeline recommended by Mortazavi et al. (Mortazavi et al. 2008). The RPM data was transformed using log2 to stabilize the variance and then performed a further quantile normalization. Approximately ~1300 transcripts were differentially expressed (> 1.3 fold difference and $P < 0.05$). The software Ingenuity Pathway Analysis (IPA, Qiagen) was used to help in the interpretation of the dataset.

### Real time quantitative PCR (RT-qPCR)

To validate transcriptomic analysis, targeted gene expression was conducted using RT-qPCR). Briefly, RNA was first extracted from liver samples using Trizol reagent (#15596018, Life Technologies) following the manufacture's recommendations, treated with DNAase, and reverse transcribed (catalog #95048-100, Quanta Biosciences). Next, the cDNA (RT products) were amplified by RT-qPCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (catalog #4312074, Life Technologies). Primers used in this study, including the 18S ribosomal housekeeping gene, are shown in Table 1. The cycling conditions for the RT-qPCR were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of a two-step amplification program with 95°C for 15 s and 58°C for 1 min. To exclude contamination with unspecific PCR products we used melting curve analysis applying the dissociation protocol from the Sequence Detection system. The $^{2-\Delta\Delta}$Ct method was chosen to establish the relative expressions of target genes in this study (Schmittgen and Livak 2008). Relative mRNA expression was obtained by normalizing CAS expression values to 1.0 for comparison with the SPI group.

Statistical evaluation

The analysis of the data was assessed with the software Graph Pad Prism version 6.00 for Windows, La Jolla California USA, and Student's t-test. Differences were considered significant at $P<0.05$.

RESULTS

RNAseq data

To validate the RNAseq dataset, RT-qPCR was conducted on 12 of the most differentially expressed genes provided by Ingenuity Pathway Analysis (IPA) in the RNAseq dataset. Fold differences in mRNA expression by RNAseq and RT-PCR for top up- and down-regulated genes in liver of the SPI-fed rats compared to CAS-fed rats are shown in Table 2. Regression analysis of mean values shown in Figure 2 indicate a reasonable agreement and validation of the

RNAseq dataset based on the correlation coefficient and P value obtained by RT-PCR and RNAseq. Removal of the one gene (PRSS8, Table 2) that exhibited the greatest difference between the two gene expression values increased the level of significance of the correlation (P=0.0003) but had no effect on the correlation coefficient (data not shown). Therefore, we feel that the data presented in Table 1 represents a viable validation of the RNAseq dataset.

RT-qPCR gene expression

The RT-qPCR analysis of mRNA expression of select genes in this study shown in Figure 3 indicate that 8 of the 12 genes selected based on RNAseq data were differentially expressed between the CAS and SPI-fed groups. Four genes that were up-regulated in the SPI group were neuronal pentraxin 2 (NPTX2), glutamic-pyruvic transaminase (GPT), indolethylamine N-methyltransferase (INMT), and histamine ammonia-lyase (HAL). There were also four genes up-regulated in the CAS-fed rats: protease serine 8 (PRSS8), Ajuba LIM protein (AJUBA), colony stimulating factor 2 receptor beta (CSF2RB), and cytochrome P450 family 2 subfamily c polypeptide 12 (Cyp2c12). Although tumor necrosis factor receptor superfamily 12A (TNFRSF12A) and Lipocalin 2 (LCN2) did not show differences between groups by qPCR, these were differentially expressed in the RNAseq data based on P value and fold difference cutoffs that had been established (see Table 2). Importantly, the direction of their expression, either up- or down-regulated, followed the results of the RNAseq analysis.

DISCUSSION

Feeding SPI can attenuate the development of liver steatosis in an obese rat model but the exact mechanisms responsible for this attenuation are not clear (Hakkak et al. 2015). Understanding what enables SPI to attenuate liver steatosis could lead to new treatments that could help control the progression of irreversible damage associated with cirrhosis. To investigate fundamental mechanisms of SPI-mediated attenuation of NAFLD, we have carried out global gene expression analysis on liver obtained from obese Zucker rats fed a CAS- or

SPI-based protein diet. A necessary first step in conducting pathway analysis of global expression data is to first validate the data by comparison to targeted gene expression analysis by PCR and is the major goal of the present study. In this study, we have selected genes that were indicated to be the most differentially expressed (up- or down-regulated) in RNAseq data obtained from rats fed CAS- or SPI-based diets. In the discussion below, differential expression of some genes is expected and associated with inflammation that is characteristic of liver steatosis but expression of other genes (e.g., NPTX2) are novel and have not been reported previously to our knowledge with respect to NAFLS or soy-based diets.

NPTX2 was up-regulated in the liver of SPI-fed rats (Table 1). NPTX2 belongs to the pentraxin family is a member of an ancient superfamily of genes that is phylogenetically highly conserved across the animal kingdom (Mantovani et al. 2008). This superfamily can be divided according to their length and mechanisms of action into short and long pentraxins. Long pentraxins, are cytokine-inducible genes or molecules expressed in different tissues, including adipocytes, monocytes, and endothelial cells. NPTX2 is a type of neuronal long pentraxin involved in excitatory synapse formation. Moreover, NPTX2 is known to be up-regulated in Parkinson's disease and in pancreatic cancer (Park et al. 2007). The proteins of the pentraxin family are in a class of pattern recognition receptors (PRRs) and therefore are involved in acute immunological responses (Gewurz et al. 1995). Pentraxins are acute phase catalysts interacting with cytokines to modulate inflammation at both tissue and systemic levels. The plasma concentration of the short pentraxin C-reactive protein, secreted by the liver, is an approved marker as a diagnostic tool of systemic inflammation in obese individuals (Barazzoni et al. 2016; Mantovani et al. 2008). However, pentraxin 3 (PTX3), which is a typical structure for long pentraxins, has been reported to limit tissue damage and the inflammatory process in several disease models, including atherosclerosis, myocardial infarction, kidney injury, and experimental carcinogenesis (Barazzoni et al. 2016; Mantovani et al. 2008). As NAFLD and obesity are associated with a general systemic inflammation due to elevated circulating levels of

proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL) 6, the increase in NPTX2 expression in SPI-fed rats might be an intrinsic mechanism that functions to attenuate systemic inflammation in NAFLD.

In addition, there are epigenetic studies focused on aging that relate several components found in the diet, such as epigallocatechin-3-gallatte (EGCG) from green tea and genistein from soybeans, that could be controlling gene expression through DNA methylation (Johnson et al. 2012). This type of DNA methylation can occur not only on the coding regions of specific genes of interest, but also on their promoters (Bacalini et al. 2014). In these studies, the gene NPTX2 is indicated as one of the most important genes under epigenetic influence (Bocklandt et al. 2011), along with tumor suppressor genes such as hMLH1 and RARb (Bacalini et al. 2014; Johnson et al. 2012). Thus, it is reasonable to hypothesize that SPI in the present study might be exerting epigenetic control over NPTX2 expression.

O'Brien et al. (O'Brien et al. 1999) reported that NPTX2 [Narp] protein expression does not occur in rat liver, yet with our study, we have detected its expression both by PCR and RNAseq. The team in the aforementioned research (O'Brien et al. 1999) could have obtained a negative result due to antibody not recognizing the appropriate epitopes for NPTX2. However, it was also reported that expression of human NPTX2 mRNA does not occur in human liver (Maffei et al. 2004). Thus, the observation of increased NPTX2 expression in SPI-fed rats may be a novel discovery in the present study.

GPT (or alanine amino transaminase) was also upregulated in the SPI-fed rat liver (Table 1). GPT plays a key role in the intermediary metabolism of glucose and amino acids. Specifically, it is an enzyme that catalyzes the reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate. Thus, GPT plays an important role in gluconeogenesis and amino acid metabolism (Figure 4) (Jadaho et al. 2004). Serum activity levels of this enzyme are routinely used as a biomarker of liver injury caused by drug toxicity,

infection, alcohol, and steatosis. Although elevated GPT is considered a biomarker of NAFLD, caused in part by insulin resistance characteristic of metabolic syndrome (Calcaterra et al. 2011), this hypothesis has been challenged due to the difficulty in establishing a direct correlation between the circulating GPT levels and the exact stage of the NAFLD. Despite the fact that GPT exists as two isoforms (GPT1 and GPT2, respectively) coded by two different genes located on different chromosomes in humans, the enzymatic activity in circulation is measured as total GPT (Sookoian et al. 2016). It is also known that both isoforms not only have different expression pattern in other tissues, but also different intracellular localizations. According to a recent study (Sookoian et al. 2016), since GPT2 is the mitochondrial isoform of this enzyme, circulating levels of GPT should be considered as sensors of global metabolic deregulation, including mitochondrial energetic control, rather than as merely a biomarker.

INMT catalyzes the N-methylation of indoles such as tryptamine and related compounds and a major mechanism for degradation of endogenous and exogenous compounds (Thompson et al. 1999). However, the function of INMT and the physiological significance of the N-methylation pathway of indolethylamine metabolism is still not clear (Kärkkäinen et al. 2005; Thompson et al. 1999). The expression of INMT mRNA has been found in several mammalian tissues, having the highest expression in the thyroid, adrenal gland, and lungs. Low or absent expression has been reported in brain, spleen, thymus, peripheral blood leukocytes, liver, and kidney (Thompson et al. 1999). It is also present in most of stromal and epithelial cells of organs related to the autonomous nervous system but absent from neurons and striated muscle cells (Kärkkäinen et al. 2005). INMT was indicated to be present in a fatty liver gene set as part of the human phenotype ontology project (Köhler et al. 2014), but what role it plays in fatty liver disease is not apparent at this time. The up-regulation of INMT in SPI-fed rats could indicate it has a role in attenuation of NAFLD.

Histidine ammonia lyase (HAL), also known as histidase, catalyzes the first reaction in the histidine degradation pathway. HAL is a cytoplasmic enzyme present in liver and skin tissue and catalyzes the oxidative deamination of L-histidine to produce urocanic acid and ammonia (Tovar et al. 2002). At the transcriptional level, HAL is regulated in the liver by the protein content in the diet. The amounts of most amino acid-degrading enzymes in the liver increase as protein intake rises and decrease as protein intake falls (Torres et al. 1998). When the histidine requirement has been met, increased HAL expression and activity enables the liver to catabolize and eliminate excess histidine after the ingestion of a high protein diet. Thus, regulating the histidine concentration in plasma by controlling the food consumption of histidine is an important role that HAL plays at the cellular and organismal level (Torres et al. 1998; Tovar et al. 2002). It was determined that both casein and soy increase hepatic HAL levels (Tovar et al. 2002). However, the groups of rats with higher intake of both proteins (casein or soy at 50% versus 18%) triggered higher levels of HAL than the groups with lower intake of the same two protein sources (Tovar et al. 2002). Gene expression of HAL has been reported to be regulated by estrogen, glucagon, and glucocorticoids generated during a catabolic state (Armstrong and Feigelsong 1980; Tovar et al. 2002). It has been demonstrated that the administration of glucagon to rats promoted the expression of HAL (Alemán et al. 1998). To our knowledge, the present study is the first to report relationships of HAL expression associated with NAFLD in SPI- and CAS-fed obese Zucker rats.

Four genes in the present study, SerpinA 6, IL-33, TNFRSF12A, and LCN2 were differentially expressed in the RNAseq data, but were not in the RT-qPCR data (Table 2, Figure 2). This is not uncommon in global expression datasets. Importantly, the direction of expression (up- or down-regulated) were similar by either analysis. Therefore, discussion of these genes will be based on the differential expression obtained in the RNAseq analysis.

SperinA 6 was elevated in liver of SPI-fed rats (Table 2). This molecule belongs to the large family of protease inhibitors called serpins. Serpin-like genes have been described in numerous phyla, from viruses to animals (Law et al. 2006). Most serpins inhibit serine proteases, but there are some rare serpins performing a non-inhibitory function. SerpinA 6 which encodes an □-globulin also called corticosteroid-binding globulin (CBG) or transcortin, is primarily produced in the liver and to a lesser extent in the placenta, kidney, endometrium, lung, pituitary, and hypothalamus (Gagliardi et al 2010; Hammond et al. 1987; Law et al. 2006). SerpinA 6 is a transporter of several anti-inflammatory steroids and progesterone in plasma (Henley and Lightman 2011). Under normal conditions in humans, about 80% to 90% of cortisol is bound to SerpinA 6 with high affinity (Gagliardi et al. 2010; Hammond et al. 1987; Richard et al. 2010). By regulating the free cortisol concentration in blood, SerpinA 6 controls the bioavailability of corticosteroids, thus acting as a buffer during a secretory surge that can increase the levels of cortisol or as a reservoir of this corticosteroid during periods of decreased secretion (Gagliardi et al. 2010; Henley and Lightman 2011).

SerpinA 6 levels in blood are associated with body mass index, insulin resistance, serum levels of interleukin-6, and adiponectin as well as proliferation and differentiation of preadipocytes (Braun et al. 2010). The role of SerpinA 6 as a simple transporter is starting to change in the last few years. SerpinA 6 is the substrate of elastase that is released in high concentration by activated neutrophils at sites of inflammation. Once elastase cleaves SerpinA 6, the binding affinity for cortisol is reduced 10-fold, releasing cortisol at the inflammation site as a result. Thus, SerpinA 6 might have a key role in preventing tissue damage during inflammation such as that occurring in NAFLD, since cortisol modulates inflammatory response (Braun et al. 2010; Gagliardi et al. 2010). The role of SerpinA 6 as an indirect anti-inflammatory molecule could shed some light on our research, partially explaining why there are no signs of inflammation on the SPI-fed rat liver.

IL-33 belongs to the IL-1 superfamily of cytokines expressed in healthy vascular endothelial cells, epithelial cells, fibroblasts and smooth muscle cells (Haraldsen et al. 2009; Miller 2011). IL-33 is expressed primarily in cells from tissues involved in the production and maintenance of a barrier. When the barriers are damaged, IL-33 behaves as an alarm, being released to initiate the acute local inflammation and tissue-repair process. IL-33 has a function as a ligand for the Th2-associated ST2 receptor activating NFkB and mitogen-activated kinases. IL-33 also functions as a nuclear factor  regulating gene transcription (Cayrol and Girard 2014; Haraldsen et al. 2009; Martin and Martin 2016; Miller 2011). IL-33 was reported to be a regulator of hepatic ischemia and reperfusion injury related with the activation of NFκB, p38 MAPK, cyclin D1, and Bcl-2 that restricts liver injury and decreases inflammation promoters in mice (Sakai et al. 2012).

In a recent review, Sun et al. (Sun et al. 2017) reported that IL-33 may serve a protective role in fatty liver disease. Pejnovic et al. (Pejnovic et al. 2016) developed an NAFLD mouse model through feeding a high fat diet. Treating these NAFLD mice fed a high fat diet with IL-33 was able to ameliorate hepatic steatosis as well as  insulin resistance and glucose intolerance (Pejnovic et al. 2016). Furthermore, treating genetically obese mice with IL-33 reduced fat accumulation (adiposity), possibly through induction of Th2-mediated cytokine production (Miller et al. 2010). Thus, it is possible that the elevation of IL-33 in SPI-fed rats played a role in the reduction of liver steatosis compared to CAS-fed rats in the present study.

There are some studies relating consumption or treatment with phytoestrogens with immune response, mostly when members of IL1 superfamily are involved, and chronic diseases and other conditions, such as asthma, inflammatory bowel diseases, and radiation-induced bone marrow failure (Ha et al. 2013; Martin and Bolling 2015; Sandoval et al. 2010; Tanaka and Takahashi 2013). Nevertheless, less is known regarding the effects of IL33 in the presence of phytoestrogens or any isoflavones in NAFLD.

PRSS8 was up-regulated in liver obtained from rats fed the CAS-based diet (Table 2, Figure 2). PRSS8, also known as prostasin and channel-activating protease 1 (CAP1), is a trypsin-like serine peptidase. PRSS8 was first identified as a secreted prostate gland product (Tong et al. 2004; Yan et al. 2014). In humans, PRSS8 mRNA expression has been detected in prostate, liver, salivary gland, kidney, lung, pancreas, colon, bronchus, and in some cells from the kidney (Chen and Chai 2012; Yu et al. 2014). Uchimura et al. reported in 2014 that upregulation of PRSS8 protected mice from chronic inflammation by reducing toll-like receptor 4 (TLR4) attachment to the endoplasmic reticulum (Uchimura et al. 2014). Furthermore, down-regulation of PRSS8 in mice fed a high fat diet contributed to hepatic insulin resistance and development of diabetes. Thus, in the present study, down regulation of PRSS8 in the SPI-fed rats may not be beneficial with respect to preventing liver steatosis. Possibly, the down regulation of PRSS8 expression in SPI-fed rats indicates there is no signal needed to suppress inflammation relative to the extensive liver steatosis present in the CAS-fed rats. Mechanistic studies will need to be conducted to verify this hypothesis.

AJUBA, also known as ajuba LIM protein, belongs to the LIM protein family, that is characterized by tandem homologous C-terminal LIM domains and a unique N-terminal preLIM region rich in glycine and proline residues (Fan et al. 2015). This arrangement contributes to the linking and/or strengthening of epithelial cell-cell junctions in part by linking adhesive receptors to the actin cytoskeleton. The LIM motif is a double zinc finger structure and functions as a protein-protein interface (Fan et al. 2015).

In humans, AJUBA expression was elevated in patients exhibiting nonalcoholic steatohepatitis (NASH) compared to patients with healthy liver, but there were no differences in expression between patients with NAFLD compared to those with healthy liver (Arendt et al. 2015). Thus, the upregulation of AJUBA in CAS-fed compared to SPI-fed rats is indicative of severe liver steatosis and concurs with the findings by Arendt et al. (Arendt et al. 2015).

A third gene that was up-regulated in CAS-fed rats (down-regulated in SPI-fed rats) was CSF2RB (Table 2, Figure 2). The CSF2RB protein is the common beta chain subunit of the high affinity receptor for Interleukin 3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor receptor (GM-CSF receptor), that are all cytokines and important regulators of hematopoiesis and inflammation (Akdis et al. 2011; Woodcock et al. 1994). Tallino et al. reported that a copper deficient diet up-regulated CSF2RB in a NAFLD mature rat model (Tallino et al. 2015). Thus, the increase in CSF2RB in CAS-fed rats in the present study agrees with Tallino et al., with CAS-fed rats exhibiting increased liver steatosis compared to SPI-fed rats.

CYP2C12, which belongs to the cytochrome P450 superfamily (CYP) of microsomal enzymes broadly present in both prokaryotes and eukaryotes and that is classified in families and subfamilies based on amino acid sequence similarities (Honkakoski and Negishi 2000; Xu et al. 2005), was upregulated in CAS-fed rats in RNAseq and RT-qPCR data (Table 2) and in shotgun proteomics (see Figure 3). The proteins of this superfamily function as mono-oxygenases, playing a major protective role from toxic and oxidative damage in the liver, intestine, kidney, and lung. CYPs act in a vast range of functions from the synthesis and metabolism of fatty acids, steroid hormones, cholesterol, bile acids, and vitamins to drug metabolism and detoxification from xenobiotics (Honkakoski and Negishi 2000; Xu et al. 2005). Since CYP expression and activity can be affected by components present in the diet which in turn can lead to changes in drug metabolism and in the development of several conditions such as cancer, cardiovascular, and fatty liver disease, there is a great abundance of studies involving the interaction between CYPs and specific dietary components (Hamilton-Reeves et al. 2007; Kishida et al. 2004; Li et al. 2007; Liu et al. 2016; Ronis 2016).

Current studies indicate that consumption of SPI protects against cancer by down-regulating the expression of CYP1 family members (Rowlands et al. 2001). There are also several reports where the expression of CYP3 and CYP4 families was activated or up-regulated

by a SPI-diet (Badger et al. 2008; Li et al. 2009; Mezei et al. 2003; Ronis et al. 2004). In a recent study, it was demonstrated that although SPI maternal consumption mitigates serum lipid levels on rat male offspring mainly through the up-regulation of important CYPs such as CYP3A1, the liver protein CYP2C12 was down-regulated in the SPI-fed group compared to the CAS-fed group (Won et al. 2017). This finding coincides with our results in this study.

The downregulation of TNFRSF12A in SPI-fed rats observed in the RNAseq data (Table 2) clearly points to a protective role of soy against development of liver steatosis based on well-known functions of this molecule. TNFRSF12A, also known as tumor necrosis factor-like weak inducer of apoptosis receptor (TWEAKR) and fibroblast growth factor inducible 14 (Fn14), is a transmembrane protein with a weak induction of apoptosis in some cell types. The human TNFRSF12A gene is expressed at relatively low levels in normal liver tissue but at high levels in liver cancer cell lines and in hepatocellular carcinoma specimens (Feng et al. 2000). Also, the expression of this protein increases in human liver tissue exhibiting NAFLD (Jakubowski et al. 2005; Wiley et al. 2001). This behavior could explain in part the fact that TNFRSF12A was down-regulated in our study in the SPI-fed rat livers that showed no sign of inflammation or fibrosis. TNFRSF12A promotes angiogenesis and the proliferation of endothelial cells and modulates cellular adhesion to matrix proteins and inhibits growth and migration in vitro (Feng et al. 2000). Nuclear factor of activated T-cells 1 (NFAT1), a type of transcription factor, regulates the expression of TNFRSF12A and its ligand, tumor necrosis factor-like weak inducer of apoptosis (TWEAK), with Lipocalin 2 to increase breast cancer cell invasion (Gaudineau et al. 2012).

Lipocalin 2 (LCN2) was upregulated in CAS-fed rats in the RNAseq data (Table 2). This molecule belongs to the heterogeneous lipocalin (LCN) family whose members display differences at the sequence level but remain similar at the structural level (Flower 1996). The LCN family is involved in the transport of small hydrophobic molecules and other various functions (Borkham-Kamphorst et al. 2013). LCN2 is a secretory glycoprotein, with different

functions ranging from transport of small molecules such as fatty acids, steroids, thyroid hormone, and retinoids, to a key role in the innate immunity, iron trafficking, and induction of apoptosis (Auguet et al. 2011; Flower 1996) . LCN2 is considered an adipocytokine (Alwahsh et al. 2014; Auguet et al. 2013) and reported to be secreted as a cytokine from the liver and kidney as well as from neutrophils, and macrophages (Esteve et al., 2009; Wang et al. 2007). Cell-based immunohistochemistry studies affirm that the main source of LCN2 in liver are the injured hepatocytes themselves (Borkham-Kamphorst et al. 2013). The presence of LCN2 protein in blood and urine is an early biomarker of acute kidney injury and other pathologic conditions (Asimakopoulou et al. 2014; Borkham-Kamphorst et al. 2013; Makris et al. 2012).

It was reported that LCN2 is likely to be one of the adipokines implicated in the pathogenesis of NAFLD since it is secreted from both adipose tissue and the liver (Auguet et al. 2013). In a diet-inducible fatty liver rat model, the researchers found a strong correlation between a high calorie diet and an increase or up-regulation of hepatic LCN2, elevated indicators of apoptosis, mitochondrial dysfunction, and lipid peroxidation in the hepatic cells (Alwahsh et al. 2014). This study was similar to ours, where LCN2 was down-regulated in the SPI-fed rats with no sign of steatosis nor inflammation.

In summary, we have validated gene expression in an RNAseq study with PCR analysis that investigated global gene expression in liver obtained from obese rats fed diets containing CAS or SPI. The SPI-fed rats became obese but exhibit less NAFLD. Within the group of genes that were targeted for validation, it is clear there are novel findings that are being reported, such as with the up-regulation of NPTX2 in SPI-fed rats. There are also genes whose transcribed proteins would be protective of liver cells based on either up- or down-regulation. Our future plan will be to take full advantage of the Ingenuity Pathway Analysis program to reveal fundamental mechanisms, i.e., pathways, upstream regulators that were differentially expressed in the two groups of rats to reveal what aspects were being altered by SPI. We also have

conducted shotgun proteomics analyses that will enable a proteogenomic picture to be developed in the prevention of NAFLD afforded by the SPI-based diet.

**Acknowledgments:** This study was supported in-part by the College of Medicine's University Medical Group (RH) and the Arkansas Biosciences Institute (WB, SD, RH). The authors wish to thank John Gregan of Arkansas Children's Research Institute and Sheena Joyner for valuable assistance with preparation of this manuscript.

**Disclosure Statement:** No competing financial interests exist.

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**Tables and Figures**

**Table 1.** Comparison of mean mRNA expression (fold difference) in liver of obese rats fed soy protein isolate (SPI) or casein (CAS) diets for 8 weeks obtained by RNAseq and RT-PCR. These mean values were used in correlation analysis (see Fig. 1).

| Gene Symbol | Gene Name | RNAseq (Fold Diff) | PCR (Fold Diff) |
|---|---|---|---|
| NPTX2 | Neuronal pentraxin 2 | 1.88* | 5.89* |
| GPT | Glutamic-pyruvic transaminase | 2.02* | 1.96* |
| INMT | Indolethylamine N- methyltransferase | 1.83 | 6.69 |
| HAL | Histidine ammonia-lyase | 1.85* | 4.33* |
| Serpina6 | Serpin family A member 6 | 1.51* | 0.38 |
| IL33 | Interleukin 33 | 1.52* | 0.66 |
| PRSS8 | Protease, Serine 8 | -5.71* | -18.52* |
| AJUBA | Ajuba LIM protein | -2.65* | -2.71* |
| CSF2RB | Colony stimulating factor 2 receptor b | -2.45* | -2.71 |
| CYP2C12 | Cytochrome P450, family 2 receptor b | -2.45* | -2.71* |
| LCN2 | Lipocalin 2 | -2.45* | -4.76 |
| TNFRF12 | TNF receptor superfamily member 12A | -2.30* | -1.96 |

[1] Values represent mean of n=8.

Positive and negative values indicate up- and down-regulation of gene expression, respectively, in livers of obese rats fed soy protein isolate-based vs casein-based diets. *Indicates significant difference ($P < 0.05$) between dietary groups.

**Table 2.** Oligonucleotide PCR primers based on the *Rattus norvegicus* genome.

| Gene | Accession No.[1] | Primer sequence | Orientation | Product Size (bp) |
|---|---|---|---|---|
| NPTX2 | NM_001034199.1 | TCCGGGCACAAGAGATCATC | Forward | 59 |
| | | GATGTTTCCAGGCATGTTCGT | Reverse | |
| GPT | NM_031039.1 | GCCCGGGAGTGGCTTT | Forward | 59 |
| | | GCAGAATGGTCATCCGGAAA | Reverse | |
| INMT | NM_001109022.1 | CCCAAAGACTACCTGACCACC | Forward | 120 |
| | | TCCCCCTACACCTCCTGTAGA | Reverse | |
| HAL | NM_017159.1 | TCATCGAGCACGTGGAACAA | Forward | 139 |
| | | ATCCAGGGCCTTACTACGGA | Reverse | |
| PRSS8 | NM_138836.1 | CCTACAATGGCGTCCACGTT | Forward | 59 |
| | | TGACACCACCCATTGATTTGA | Reverse | |
| Ajuba | NM_053503.1 | AGGCCATGGGGAAGTCCTAT | Forward | 137 |
| | | CGGGGCGTAATTTTTGTGGT | Reverse | |
| CSF2RB | NM_133555.1 | CCCAAAGCTGGGGAGAAGAAA | Forward | 126 |
| | | AACAGAGACGGTGTACTGGC | Reverse | |
| Cyp2c12 | NM_031572.1 | TTCTCAGCAGGAAAACGGAAATG | Forward | 122 |
| | | TCGATGTCCTTTGGATCAGACAG | Reverse | |
| Serpina 6 | NM_001009663.1 | TCCTCAGAGGCATATGGGAACT | Forward | 144 |
| | | AGGGGAAGACTGAGTCACGA | Reverse | |
| IL33 | NM_001014166.1 | CAGAATCTTGTGCCCTGAGC | Forward | 124 |
| | | CGGAGTAGCACCTTATCTTTTTCT | Reverse | |
| TNFRSF12A | NM_181086.3 | ACTTCAGGATGCTATGGCCC | Forward | 132 |
| | | CAGTCTCCTCTATGGGGGTAGTAA | Reverse | |
| LCN2 | NM_130741.1 | CAAGTGGCCGACACTGACTA | Forward | 105 |
| | | CCCCTTGGTTCTTCCGTACA | Reverse | |
| 18S | NR_046237.1 | AGTCCCTGCCCTTTGTACACA | Forward | 60 |
| | | GCCTCACTAAACCATCCAATCG | Reverse | |

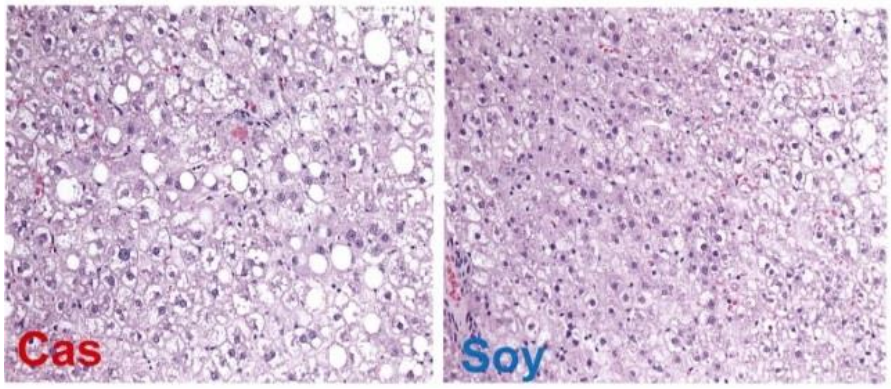


**Figure 1. Liver histology.** Obese Casein-fed rats (Cas) versus Obese Soy-fed rats (Soy) for 8 weeks Liver steatosis.

**Figure 2.** Regression analysis of mean values obtained by PCR and by RNAseq analysis.

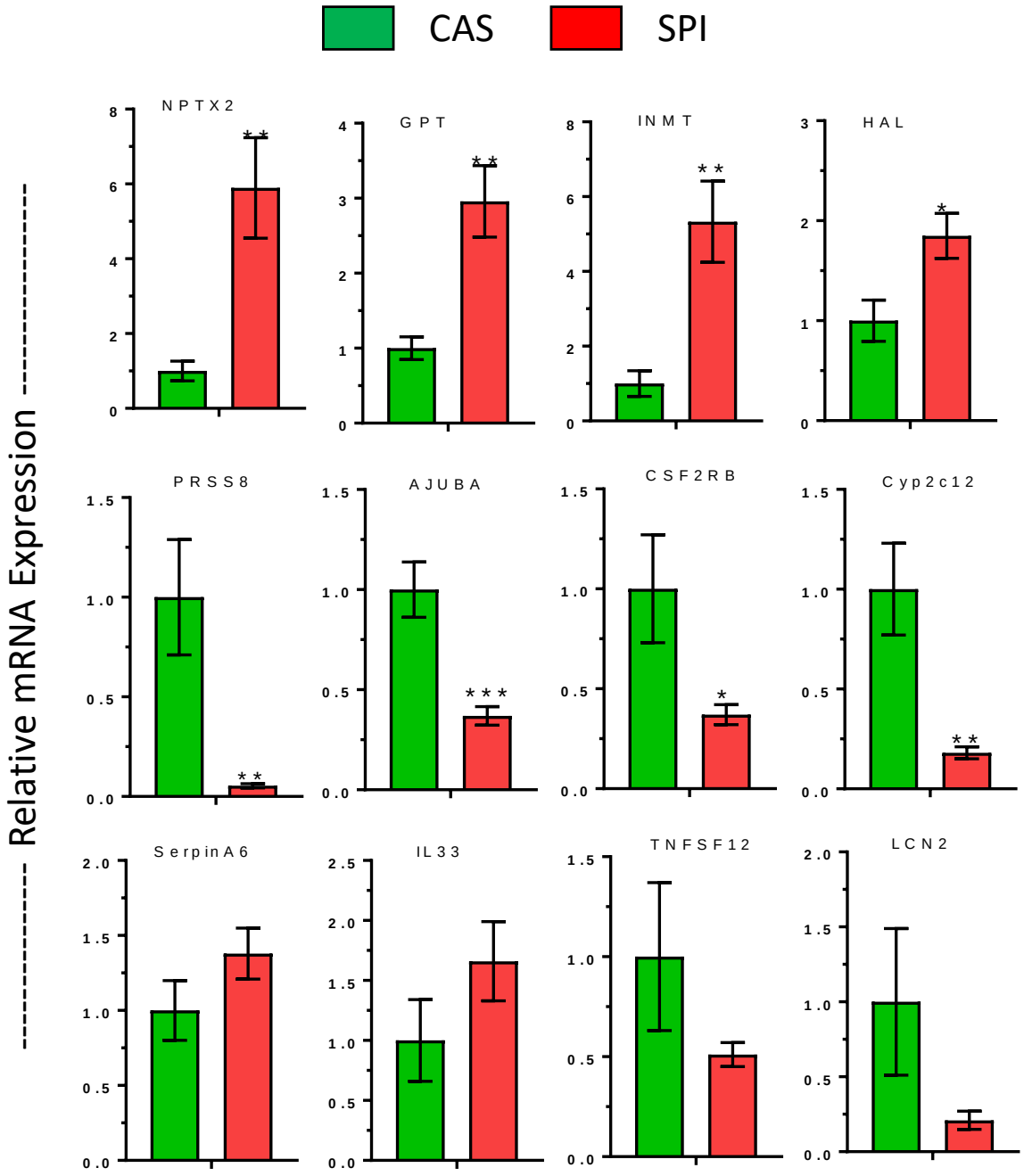


**Figure 3.** Targeted mRNA expression (RT-PCR) of genes listed in Table 1 determined in the liver of Zucker rats fed diets containing casein (CAS) or soy protein isolate (SPI) for 8 weeks. Bars represent mean ± SE (n=8); * P < 0.05, ** P < 0.01, *** P < 0.001

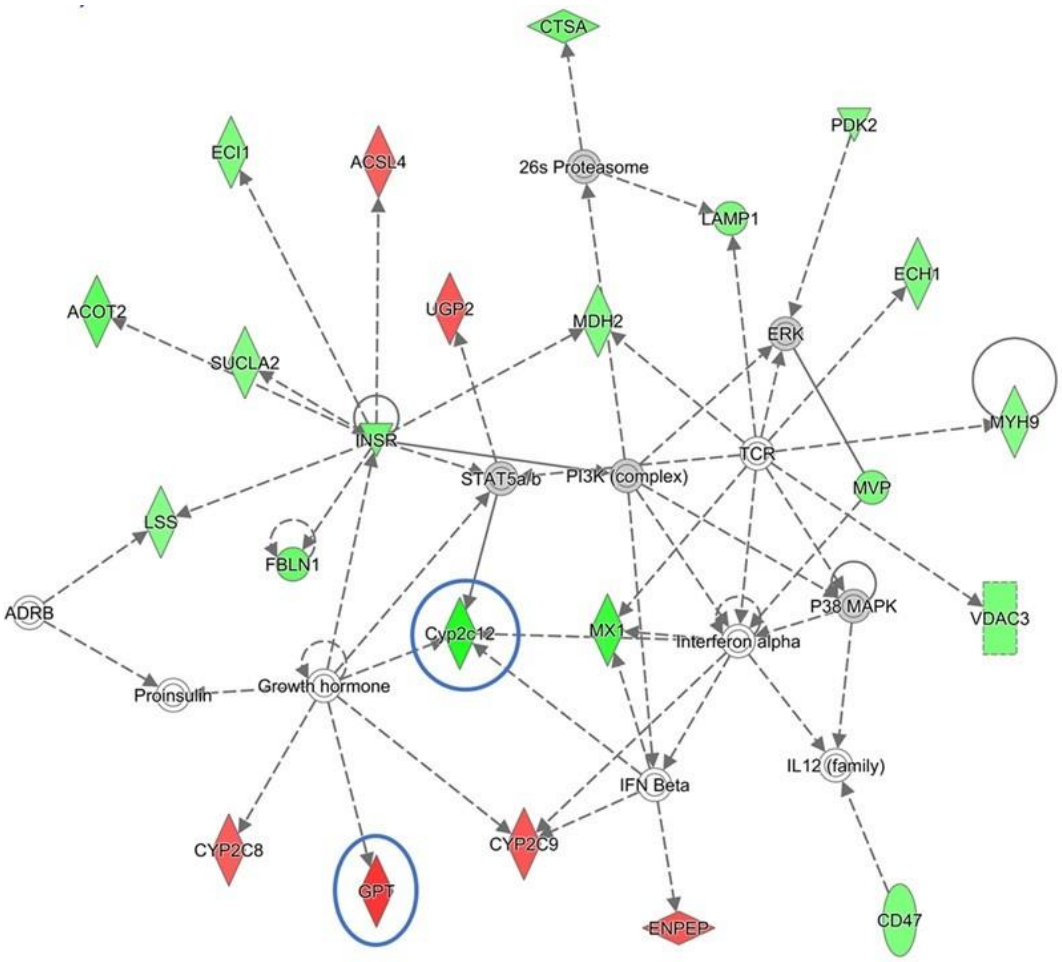


**Figure. 4** Energy Production, Lipid Metabolism, and Small Molecule Biochemistry Network built with IPA program from shotgun proteomics data (Kozaczek et al. (Kozaczek et al. 2018), unpublished) that was determined on liver samples from the same SPI- and CAS-fed animals as described in the present study. Proteins in red were up-regulated in the SPI-fed rats and proteins in green were up-regulated in the CAS-fed rats. The blue circles highlight two molecules, GPT and CYP2C12, that exhibited differential protein expression (1.3-fold difference, $P < 0.05$) concurs with RNAseq and RT-qPCR data. Molecules in gray were detected but were below the cut off values of fold difference and $P < 0.05$. Molecules in white are part of the network but were not detected in the proteomics data. The network shows their interactions with other proteins from the dataset.

## CHAPTER 4

Long-Term Soy Protein Isolate Intake Reduces Liver Steatosis by Changing Global Gene Expression in Obese Zucker Rats

**Targeted Journal will be: Frontiers of Nutrition**

## 4.1 Abstract

To understand how soy protein isolate (SPI) reduced liver steatosis in male obese Zucker rats, we conducted global gene expression (RNAseq) analysis on liver samples of male rats fed either the SPI or a control casein (CAS)-based diet (n=8 per group) for 16 wks. Liver transcriptomic was analyzed using an Ilumina HiSeq system with 100 base paired end reads for sequencing. Bioinformatics was conducted using Ingenuity Pathway Analysis (IPA) software (Qiagen, CA) with $P < 0.05$ and 1.3 fold differential expression cutoff values. Regression analysis between RNAseq data and targeted mRNA expression analysis of 12 top differentially expressed genes (from the IPA program) using quantitative PCR (qPCR) revealed a significant regression analysis ($r^2=0.55$, $P< 0.001$). In addition, all qPCR values had qualitatively similar direction of up- or down-regulation compared to the RNAseq transcriptomic data. An assessment of diseases and functions based on differentially expressed target molecules in the dataset revealed that lipid metabolism was predicted to be enhanced whereas inflammatory response was predicted to be inhibited in SPI-fed compared to CAS-fed rats at 16 weeks. Using the upstream regulator analysis and regulator effects functions of the IPA program enables the prediction of a number of upstream regulators (e.g. transcription regulators) that could be playing important roles in attenuating or promoting liver steatosis due to SPI or CAS diets. Examples of upstream regulators that were predicted to be activated (based on expression of down-stream target molecules) that were linked to increased conversion of lipid and transport of lipid in SPI-fed rats included hepatocyte nuclear factor 4 alpha and aryl hydrocarbon receptor. Examples of upstream regulators that were predicated to be activated in CAS-fed rats that were linked to predicted activation of phagocytosis and neutrophil chemotaxis included colony stimulating factor 2 and tumor necrosis factor. The results provide clear indication that long-term SPI-fed rats exhibited diminished inflammatory response and increased lipid transport in liver compared to CAS-fed rats that likely would contribute to reduced liver steatosis in this obese Zucker rat model.

## 4.2 Introduction

Excess accumulation of fat in the liver, liver steatosis, a condition strongly linked to obesity, can be divided in two major categories: Alcoholic Fatty Liver Disease and Non-Alcoholic Fatty Liver Disease (NAFLD). Alcoholic liver disease occurs due to alcohol abuse over extended period of time (Szabo, 2015), whereas NAFLD can be described as an accumulation of lipid in the liver cells that is not provoked by alcohol intake (Castaño-Rodríguez et al., 2017). In the last decade, NAFLD has gained more attention due to its high connection with other disorders, such us an increase in the lipids in the circulation, excess body weight, insulin resistance, and inflammation, diabetes type II, and vascular disorders (Castaño-Rodríguez et al., 2017; Romero-Gómez et al., 2017) that when present at the same time contribute to a larger disorder called Metabolic Syndrome. Metabolic syndrome has its origin in obesity with hereditary components and sedentary behavior (Hales et al., 2017).

The mild steatosis in NAFLD if left untreated can lead to non-alcoholic steatohepatitis (NASH), and finally to irreversible damage to the liver due to cirrhosis. The development of NAFLD takes place in two stages; Stage 1) Insulin resistance develops that is accompanied by lipid accumulation in the liver in the form of triglyceride, and Stage 2) Mitochondrial dysfunction with mitochondrial reactive oxygen species production promotes oxidative stress leading to inflammation and hepatic fibrosis (Feng et al., 2017; Musso et al., 2012). Recently, a revision of the progression of pathogenesis has been proposed in which NAFLD can be tentatively diagnosed in patients with elevated liver enzymes and by imaging in the absence of other causes of liver disease. However, a definitive diagnosis can only be made by liver biopsy (Serviddio et al., 2008; Weiß et al., 2014). Estimations of the prevalence suggest that NAFLD could be the most common form of chronic liver disease in adults in the US, Asia, Australia, and Europe that may affect 10% to 35% of the worldwide population (Bellentani and Marino, 2009). There is also increasing concern of NAFLD as a significant form of liver disease in pediatric populations (Cornier et al., 2008; Kleiner et al., 2005).

Although short-term feeding a diet containing soy protein isolate (SPI) specifically targeted and halted the development of liver steatosis in male and Female obese Zucker rats compared to those fed a casein (CAS)-based diet (Hakkak et al., 2015), the exact mechanisms responsible for SPI-mediated amelioration of liver steatosis is not understood. To better understand fundamental mechanisms by which SPI attenuated NAFLD, we have conducted global gene expression (RNA seq) analysis on liver tissue obtained from obese rats fed the SPI- and CAS-based diets for 8 weeks (Kozaczek et al., 2019). The results of this study indicated gene expression favoring or promoting anti-inflammatory activities in SPI-fed rats and inflammatory activities in CAS-fed rats and consistent with the observation of reduced liver steatosis in SPI-fed rats (Hakkak et al., 2015). In the present study, we report on global expression analysis of liver obtained after long term (16 weeks of feeding CAS- and SPI-based diets) to obese rats to intensify efforts to reveal fundamental mechanisms associated with development of liver steatosis and amelioration of liver steatosis afforded to obese rats consuming the SPI-based diet.

## 4.3 Materials and Methods

### 4.3.1. Experimental Design

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences (Protocol code number 3242; approved on 12/6/2011). Liver tissue was obtained from male obese Zucker rats from a previous study (Hakkak et al. 2015). Briefly, rats (6 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). After one week of acclimation, rats were randomly assigned to either a casein (CAS) diet or soy protein isolate (SPI) diet. They were housed in individual cages and provided the diets ad libitum for 16 weeks and all rats were humanely killed, and liver samples obtained, and flash frozen in liquid nitrogen and stored at −80°C.

4.3.2. Transcriptomic Analysis

A phenol-chloroform solution was used to extract RNA from liver samples. A 1% agarose gel was used to evaluate the quality of RNA and concentrations were assessed with Take 3 micro volume plate with a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). RNA sequencing (RNAseq) of RNA samples was conducted using an Illumina HiSeq 100 base pair paired end read at the Research Support Facility (Michigan State University, East Lansing, MI). The CLC Genomics Workbench 8 software that incorporates the pipeline recommended by Mortazavi et al. (2008) was used to map the reads to *Rattus rattus* genome assembly (version 4). The RPM data was transformed using log2 to stabilize the variance and then performed a further quantile normalization. Over 1200 transcripts were differentially expressed (> 1.3 fold difference and $P < 0.05$). Ingenuity Pathway Analysis (IPA) commercial software (Qiagen, CA) was used to help in the interpretation of the dataset.

4.3.3. Real time quantitative PCR (RT-qPCR)

Targeted gene expression was conducted using RT-qPCR) to validate transcriptomic results. Briefly, RNA was extracted from liver samples using Trizol reagent (#15596018, Life Technologies) following the manufacture's recommendations, treated with DNAase, and reverse transcribed (catalog #95048-100, Quanta Biosciences). Next, the cDNA (RT products) were amplified by RT-qPCR (Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green Master Mix (catalog #4312074, Life Technologies). Primers used in this study, including the 18S ribosomal housekeeping gene, are shown in Table 1. The cycling conditions for the RT-qPCR were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of a two-step amplification program with 95°C for 15 s and 58°C for 1 min. To exclude contamination with unspecific PCR products we used melting curve analysis applying the dissociation protocol from the Sequence Detection system. The $^{2-\Delta\Delta}$Ct method was chosen to establish the relative expressions of target genes in this study (Schmittgen and Livak, 2008). Relative mRNA

expression was obtained by normalizing CAS expression values to 1.0 for comparison with the SPI group.

4.3.4. Statistical evaluation

The analysis of qPCR data and regression analysis between qPCR and transcriptomic (RNAseq) was assessed with the software Graph Pad Prism version 6.00 for Windows, La Jolla California USA, and Student's t-test. Differences were considered significant at $P<0.05$.

Upstream regulator analysis by IPA is based a combination of a) the number and degree of differential expression of downstream target molecules in the existing dataset, and b) on prior knowledge of expected effects between transcriptional regulators and their target genes from published literature citations that have been curated and stored in the IPA program. Upstream regulator analysis determines how many known targets or regulators are within the user's dataset and compares each differentially expressed molecule to the reported relationship in the literature. If the observed direction of change is mostly consistent with either activation or inhibition of the transcriptional regulator, then a prediction is made, and an activation z score generated that is also based on literature-derived regulation direction (i.e. "activating" or "inhibiting"). Activation z scores > 2.0 indicate that a molecule is activated whereas activation z scores of < -2.0 indicate that a target molecule is inhibited. The p-value of overlap measures whether there is a statistically significant overlap between the dataset molecules and those regulated by an upstream regulator is calculated using Fisher's Exact Test, and significance is attributed to p-values < 0.05.

4.4 Results

4.4.1. Top differentially expressed genes: RT-qPCR and RNAseq data

RT-qPCR was conducted on 13 of the most differentially expressed genes provided by Ingenuity Pathway Analysis (IPA) in the RNAseq dataset included NPTX2, IL33, PRSS32, Resp18, RGN, SULT2A1, AMDHD1 that were up-regulated, and Gnai, PRSS8. Cidea, MAGEE1, Sdr16c6 and HIVEP2 that were down-regulated in the SPI-fed compared to CAS-fed

rats. Fold differences in mRNA expression by RNAseq and RT-PCR for top up- and down-regulated genes in liver of the SPI-fed rats compared to CAS-fed rats are shown in Table 2. Regression analysis of mean values shown in Figure 1 indicate that there was a significant correlation ($r^2 = 0.55$, $P < 0.001$) between targeted PCR and RNAseq data expression in the most differentially expressed genes in the transcriptomic dataset.

4.4.2 Upstream Regulators

Predictions of activation or inhibition of upstream regulators provide insight into potential mechanisms in this study by which SPI feeding ameliorates liver steatosis in NAFLD. A partial list of upstream regulators predicted to be activated or inhibited from the RNAseq data obtained in SPI-fed rats vs CAS-fed obese rats after 16 weeks of feeding is presented in Table 3. Top upstream regulators predicted to inhibited in SPI-fed rats include tumor necrosis factor (TNF), Interferon Gamma (IFNG), colony stimulating factor 2 (CSF2), resistin-like beta (RETNLB) and TNF receptor 1 beta (TNFRSF1B) (Table 3A). Top upstream regulator genes predicted to be activated in SPI-fed rats include Acyl-CoA (ACOX1), hepatocyte nuclear factor 4 alpha (HNF4A), insulin induced gene 1 (INSIG1), HNF1 homeobox A (HNF1A), immunoglobulin G (IgG), and Aryl hydrocarbon receptor (AHR). A complete list of upstream regulators predicted to be inhibited or activated in the SPI-fed rats is provided in Supplementary Table 1A and 1B, respectively. There were 32 genes predicted to be inhibited in SPI-fed rats (Supplementary Table 1A) and 15 genes that were predicted to be activated in the SPI-fed rats (Supplementary Table 1B). Each of these predictions for upstream regulators point toward fundamental mechanisms that are involved in attenuation of liver steatosis provided by feeding the SPI to the obese rats.

A heat map of the RNAseq data generated by IPA software is presented in Figure 2. Blue represents diseases and functions that were predicted to be inhibited whereas orange represents those predicted to be activated in the SPI-fed vs. CAS-fed rats. The darker the color the stronger to prediction based on activation Z-scores calculated by the Ingenuity Pathway

Analysis program. A breakdown of specific functions predicted to be activated or inhibited in the dataset (with activation Z-scores of < -2.0 or > 2.0, respectively) within two broad diseases and functions classification (lipid metabolism and inflammatory response) is presented in Figure 3. Each of the specific processes listed with lipid metabolism would be predicted to be enhanced in the SPI-fed rats whereas each of the processes listed under inflammatory response would be predicted to be inhibited in the SPI-fed rats. The list of functions under lipid metabolism is consistent with the observation of reduced liver steatosis in the SPI- compared to CAS-fed rats. Furthermore, the list provided under inflammatory response clearly indicates that inflammation would be inhibited in the SPI-fed rats.

The activation Z-scores of processes presented in Figure 3 were calculated by the IPA program based on differential expression of genes in the dataset and on the relationships of gene expression with the function reported in the scientific literature. An example of one of the networks generated by the IPA program analysis of the dataset for efflux of cholesterol is presented in Figure 4A. The IPA program can also provide predictions of activation or inhibition of upstream transcription factors in regulator networks that connect transcription factors to specific diseases or function through differentially expressed molecules in the functional network. In the example shown in Figure 4B, the transcription factor RETNLB (restin-like protein beta) was predicted to be inhibited in SPI- compared to CAS-fed rats. Additional examples of regulator networks are presented in Figures 5 and 6. In Figure 5, hepatocyte nuclear factor one (HNF1) homeobox A (HNF1A) and HNF4A are transcription factors predicted to be activated in the SPI-fed rats that would contribute to lipid transport from the liver. Two regulator networks indicating that phagocytosis and chemotaxis of neutrophils are predicted to be inhibited in SPI-fed rats through down-regulation (inhibition) of colony stimulating factor 2 (CSF2) and tumor necrosis factor (TNF) are shown in Figure 6.

## 4.5 Discussion

NAFLD is characterized by an unhealthy accumulation of lipids within hepatocytes accompanied by inflammation of the liver tissue. In this study, we demonstrated that a long-term feeding of SPI enhances lipid metabolism and decreases several components of inflammation response (Fig. 3). Several functions that were predicted to activated (e.g. lipid transport, efflux, and conversion of lipid) in SPI-fed rats are processes that could reduce lipid accumulation in hepatocytes. Similarly, processes and functions involved in inflammatory response such as phagocytosis, chemotaxis of neutrophils, and immune response of cells, were inhibited in SPI-fed rats thus ameliorating NAFLD symptoms. A wide range of molecules were predicted to be activated or inhibited in liver of obese rats fed an SPI- compared to CAS-based diet. As discussed below, these predictions point towards mechanisms by which SPI ameliorates liver steatosis and can serve as hypotheses to be tested in future studies.

Acyl-CoA Oxidase 1 (ACOX1) (Table 3), also called Peroxisomal acyl-coenzyme A oxidase 1, is a transcription factor predicted to be activated (z score = 4.04) in liver of rats provided a SPI diet. Unlike most medium and long chain fatty acids whose oxidation occurs in the mitochondria, very long fatty acids are degraded in peroxisomes (Vluggens et al., 2010). ACOX1 catalyzes the first step of the peroxisomal beta-oxidation of very long fatty acids in the fatty acid degradation pathway (Zuo et al., 2007). A study with a point mutation of ACOX1 showed that beta-oxidation provides a clear link between fat metabolism and immune responsiveness (Moreno-Fernandez et al., 2018). In this study, the authors found an increase in the hepatic neutrophil infiltration when ACOX1 was not functioning properly. This finding is consistent with the results in our study predicting decreased hepatic migration and chemotaxis of neutrophils (Fig. 3 and 6B) when ACOX1 is activated in SPI treatment. In addition, a recent study concluded that ACOX1 is the post transcriptional target of miR-222 (Wang et al., 2019), a microRNA already being investigated in relationship with NASH, cancer and inflammatory diseases (Song et al., 2017); however, not strongly related to NAFLD as with NASH and HCC

(de Conti et al., 2017). The aforementioned research found that when miR-222 targets ACOX1 it inhibits it, and accumulation of triglycerides is promoted (Wang et al., 2019). These results concur with findings in the present study in which ACOX1 is predicted to be highly activated in SPI treatment; thus, preventing accumulation of triglycerides and inflammation. Since many microRNAs are involved in the pathology of insulin resistance, NAFLD, and fibrosis (Su et al., 2018) it would be reasonable to link a decreased expression or inhibition of miR-222 by the consumption of a diet high in soy proteins, although the mechanisms remain unknown. Further research its needed.

Both Hepatocyte Nuclear Factor Alpha 4 (HNF4A) and 1 (HNF1A) (Table 3B) are upstream regulators predicted by IPA program to be strongly activated in our study (z score = 3.44 and 2.55 respectively) in the liver of rats provided a SPI diet. HNF4A is a nuclear transcription factor known to regulate several hepatic genes including the related transcription factor HNF1A (Figure 5) and other genes related to lipid metabolism (Krapivner et al., 2010). There is evidence supporting HNF4A as a universal transcription regulator of hepatic cytochrome P450 (CYP) genes (Jover et al., 2001). HNF1A is mainly expressed in liver but also in pancreas and the kidneys (Tan et al., 2019). HNF4A and HNF1A are major promoters of hepatic differentiation and maturation (Deng et al., 2014; Patitucci et al., 2017). Involved in lipid metabolism, HNF4A has been demonstrated to be a central gene in the network of NASH connected to metabolic diseases (Baciu et al. 2017). Mutations in this gene have also been linked with both diabetes type 2 and maturity onset diabetes of the young (MODY) (Yamagata, 2014). An amplification of this gene has been found to be associated with colorectal cancer (Zhang et al., 2014). In the early 2000s, Lazarevich et al., not only found a strong correlation between the downregulation or lack of function of HNF4A and the progression of HCC, but also that restoration of HNF4A expression could reverse the HCC phenotype in a mouse model (Lazarevich et al., 2004). In 2010, Ning et al., established that the forced expression of HNF4A inhibits HCC and alleviates hepatic fibrosis probably through the repression of beta-catenin

signaling pathway (Ning et al., 2010), and proposed the administration of HNF4A as a possible future pathological treatment (Ning et al., 2010). The search for biomarkers for NAFLD derived HCC has shown the same trend (Frades et al., 2015): an uncoupling of important transcription factors including HNF4A. Our study predicts HNF4A to be highly expressed in SPI treatment, along with the upregulation of several CYPs and apolipoproteins (Figure 5), which could be exerting a protective influence against lipid accumulation in the liver tissue; thus, preventing inflammation and the progression to NAFLD in the long-term.

Several components of inflammatory response were predicted to be inhibited in SPI-fed rats compared with the control. For example, tumor necrosis factor (TNF) (Fig. 6 and Table 3), is an upstream regulator predicted to be inhibited (z score = -3.37) in the livers of SPI-fed rats. TNF is a pro-inflammatory cytokine secreted by a wide range of cell types able to be released as a consequence of inflammatory stimuli (Walsh et al., 1991). This protein also promotes apoptosis and necrosis, and is reactive towards endotoxins and bacterial lipopolysaccharide (LPS) (Yang et al., 1997; Zhang et al., 2010), as it can be inferred from the direct effect TNF has over lipopolysaccharide binding protein (LBP, Figure 6B). There are many ways TNF contributes to NAFLD and NASH pathophysiology. TNF is a key promoter of other inflammatory cytokines and molecules related with hepatic steatosis and fibrosis (Kakino et al., 2018). It has been suggested that TNF increases hepatic fat deposition via activation or upregulation of sterol regulatory element binding protein-1c (SREBP-1c) which in turn regulates fatty acid synthase (FAS) (Endo et al., 2007). In addition, TNF has the ability to lower the activity of the insulin receptor that contributes to the development of insulin resistance (Hotamisligil et al., 1996). In addition, inhibition of TNF in NASH patients has been reported to be beneficial either ameliorating or reversing the pathology (Satapathy et al., 2004). In a four year follow up study, TNF serum levels were reported to be increased in NAFLD patients, and associated with the stage of the disease in concordance with the literature (Seo et al., 2013). Our study not only predicts a strong inhibition of TNF under a SPI diet (Figure 6B) but also the chemotaxis of

neutrophils as a whole function is predicted to be inhibited. All the genes involved in this network were found to be downregulated in this study (See Figure 6B for a full list of the genes). Conversely, in the CAS-fed rat livers TNF might promote the chemotaxis of neutrophils by directly participating in the upregulation of the same genes. Nevertheless, the underlying mechanisms are not completely understood.

Colony stimulating factor 2 (CSF2) (Table 3A and Figure 6A), also known as granulocyte-macrophage colony-stimulating factor (GM-CSF) was predicted to be inhibited in SPI treatment at 16 weeks (activation z score = -2.29). Its receptor, CSF2 receptor beta (CSF2RB) was found to be down-regulated at 8 weeks of SPI treatment (Kozaczek et al., 2019). CSF2 is a cytokine involved in inflammatory response that modulates the production and differentiation of macrophages and granulocytes (Arcuri et al., 2009; Francisco-Cruz et al., 2014). Morrison et al. (2018) found a correlation of the up-regulation of CSF2 in humans and Ldlr-/-.Leiden mice with NASH (Morrison et al., 2018). Thus, our study concurs with Morrison et al. (2018) since CSF2 was predicted to be inhibited in the liver of rats fed the SPI diet (Figure 6A) in a pathological state previous to NASH. Conversely, our study predicts CSF2 to be highly activated in the CAS-fed rats, which presented inflammation in liver tissue, and directly promoting phagocytosis through the activation of important target molecules (see Figure 6A).

## 4.6 Conclusions

The results of the analysis conducted in this study provide a clear indication that long-term SPI feeding attenuates liver steatosis by enhancing fat metabolism and lipid transport from the liver while simultaneously lowering activity of upstream regulator genes that would inhibit inflammation. Each of the predictions of activation or inhibition of genes that were calculated from expression of downstream molecules and literature citations of similar or dissimilar relationships should be considered as hypotheses to be tested in future studies.

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4.8 Tables and Figures

**Table 1.** Oligonucleotide PCR primers based on the *Rattus norvegicus* genome.

| Gene | Accession No.[1] | Primer sequence | Orientation | Product Size (bp) |
|---|---|---|---|---|
| NPTX2 | NM_001034199.1 | TCCGGGCACAAGAGATCATC | Forward | 59 |
| | | GATGTTTCCAGGCATGTTCGT | Reverse | |
| Resp18 | NM_019278.1 | GCAGCGACATAAATGCCCAC | Forward | 136 |
| | | CAGAACATGCCTTGGGGTACA | Reverse | |
| RGN | NM_031546.1 | AGCGAGTTGGTGTAGATGCC | Forward | 83 |
| | | GAACTTGGTTCCAATGGTGGC | Reverse | |
| SULT2A1 | NM_131903.1 | GAGCTGGATTTGGTCCTCAAGT | Forward | 134 |
| | | CAGTCCCCAATAGTGCCTTTCC | Reverse | |
| PRSS32 | NM_001106983.1 | CACAAATCAACCGCTCCCAC | Forward | 127 |
| | | TTCGAGAATGACCTGCTCCG | Reverse | |
| AMDHD1 | NM_001191781.1 | GTGGGCACTGATGGGCTTAT | Forward | 123 |
| | | CACCAAACCTGGCAAGATGC | Reverse | |
| IL33 | NM_001014166.1 | CAGAATCTTGTGCCCTGAGC | Forward | 124 |
| | | CGGAGTAGCACCTTATCTTTTTCT | Reverse | |
| Cidea | NM_001170467.1 | AGGCCTTGTTAAGGAGTCTGC | Forward | 84 |
| | | CATAAGCGCCCGCATAAACC | Reverse | |
| PRSS8 | NM_138836.1 | CCTACAATGGCGTCCACGTT | Forward | 59 |
| | | TGACACCACCCATTGATTTGA | Reverse | |
| Gnai1 | NM_013145.1 | TGCAAGCCTGCTTCAACAGA | Forward | 70 |
| | | AAGTCATTCAGGTAGTACGCCG | Reverse | |
| HIVEP2 | NM_024137.1 | TACACTCTGGCTGCTATGCAC | Forward | 93 |
| | | GGGTGCATCAGGTTTCATCTGT | Reverse | |
| Magee1 | NM_001079891.1 | CCCACCTGGAGTGCATCTTT | Forward | 134 |
| | | GCCCATCTTTGGCCCATTTG | Reverse | |
| Sdr16c6 | NM_001109356.1 | GCCATCTCTCACTTCTGGATTTG | Forward | 101 |
| | | CCAACGACTCCTGCTATGCT | Reverse | |
| 18S | NR_046237.1 | AGTCCCTGCCCTTTGTACACA | Forward | 60 |
| | | GCCTCACTAAACCATCCAATCG | Reverse | |

**Table 2.** Comparison of mean mRNA expression (fold difference) in liver of obese rats fed soy protein isolate (SPI) or casein (CAS) diets for 16 weeks obtained by RNAseq and RT-PCR. These mean values were used in correlation analysis (see Fig. 1).

| Gene Symbol | Gene Name | RNAseq (Fold Diff) | PCR (Fold Diff) |
|---|---|---|---|
| NPTX2 | Neuronal pentraxin 2 | 3.80 | 7.01 |
| PRSS32 | Protease, Serine 32 | 2.28 | 1.50 |
| Resp18 | Regulated Endocrine Specific Protein 18 | 2.22 | 1.00 |
| RGN | Regucalcin | 2.22 | 1.29 |
| SULT2A1 | Sulfotransferase Family 2A Member 1 | 2.07 | 7.98 |
| AMDHD1 | Amidohydrolase Domain Containing 1 | 2.04 | 4.94 |
| Gnai | G Protein Subunit Alpha I1 | -3.24 | -2.79 |
| PRSS8 | Protease, Serine 8 | -3.09 | -10.81 |
| Cidea | Cell Death Inducing DFFA Like Effector A | -2.65 | -7.68 |
| MAGEE1 | MAGE Family Member E1 | -2.60 | -1.03 |
| Sdr16c6 | Short Chain Dehydrogenase/Reductase Family 16C Member 6 | -2.55 | -3.10 |
| HIVEP2 | Human Immunodeficiency Virus Type I Enhancer Binding Protein 2 | -2.46 | -2.65 |

[1] Values represent mean of n=8. Positive and negative values indicate up- and down-regulation of gene expression, respectively, in livers of obese rats fed soy protein isolate-based vs casein-based diets. *Indicates significant difference ($P < 0.05$) between dietary groups

**Table 3.** A partial list of upstream regulators that were predicted to be inhibited (A) or activated (B) in liver of rats provided a diet with soy protein isolate (SPI) compared to those consuming a Casein-based diet for 16 weeks.

A

| Upstream Regulator | Name | Molecule Type | Activation z-score |
|---|---|---|---|
| TNF | Tumor necrosis factor | Cytokine | -3.37 |
| IFNG | Interferon Gamma | Cytokine | -3.30 |
| CSF2 | Colony Stimulating Factor 2 | Cytokine | -2.29 |
| RETNLB | Restin like beta | Other | -2.14 |
| TNFRSF1B | Tumor necrosis factor receptor 1 beta | transmembrane receptor | -2.00 |

B

| Upstream Regulator | Name | Molecule Type | Activation z-score |
|---|---|---|---|
| ACOX1 | Acyl-CoA oxidase 1 | Enzyme | 4.04 |
| HNF4A | Hepatocyte nuclear factor 4 alpha | transcription regulator | 3.44 |
| INSIG1 | Insulin induced gene 1 | Other | 2.71 |
| HNF1A | Hepatocyte nuclear factor 1 homeobox A | transcription regulator | 2.55 |
| IgG | Immunoglobulin G | Complex | 2.17 |
| AHR | Aryl hydrocarbon receptor | ligand-dependent nuclear receptor | 2.15 |

**Figure 1.** Regression analysis of RNAseq and PCR for targeted gene expression shown in Table 2.

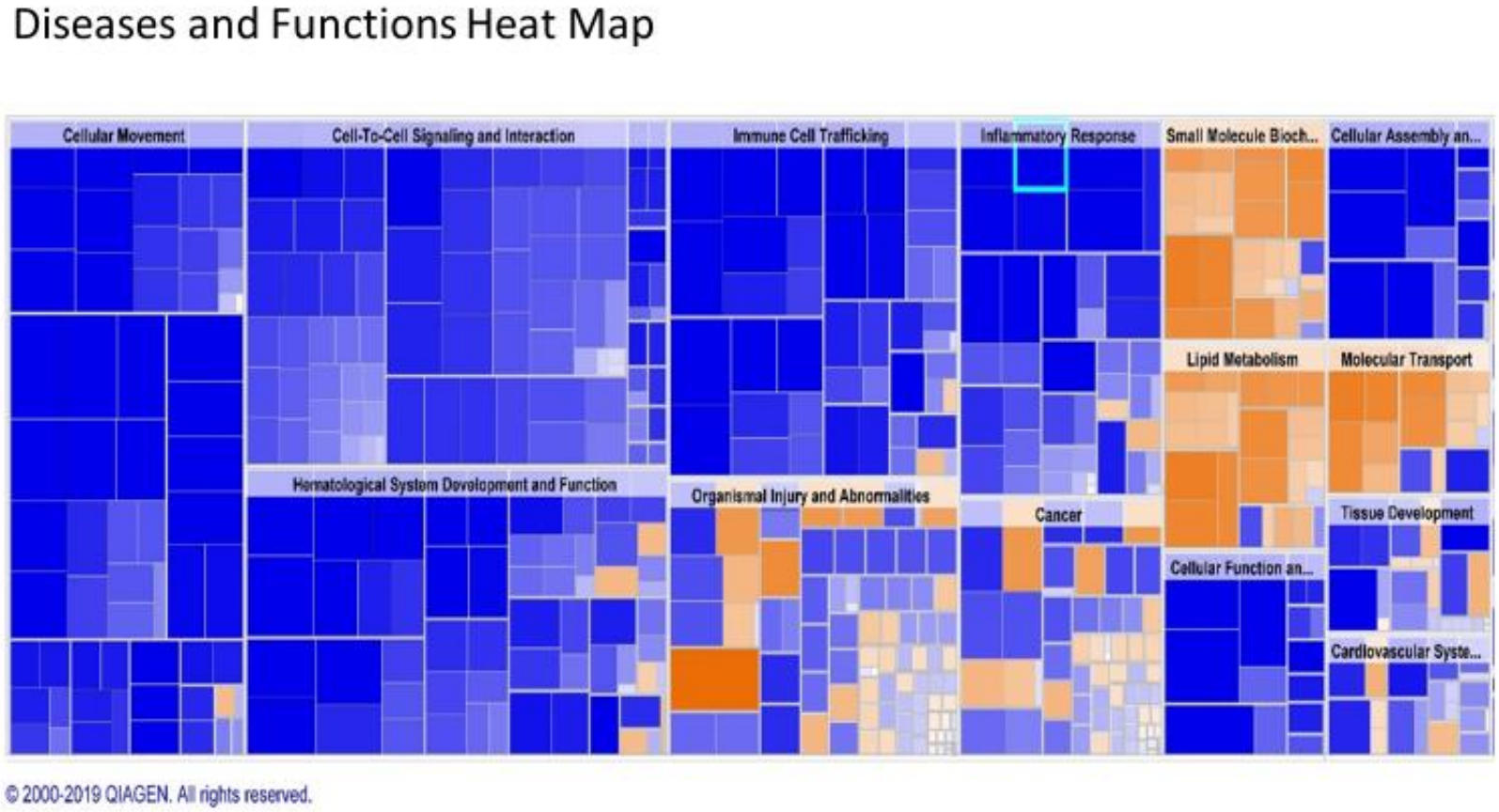


**Figure 2.** Heat map of diseases and functions arranged by Z-score with the IPA program. Functions in blue indicate inhibition while those in orange indicate activation in SPI vs CAS-fed rats. Darker colors indicate greater activation or inhibition of function.

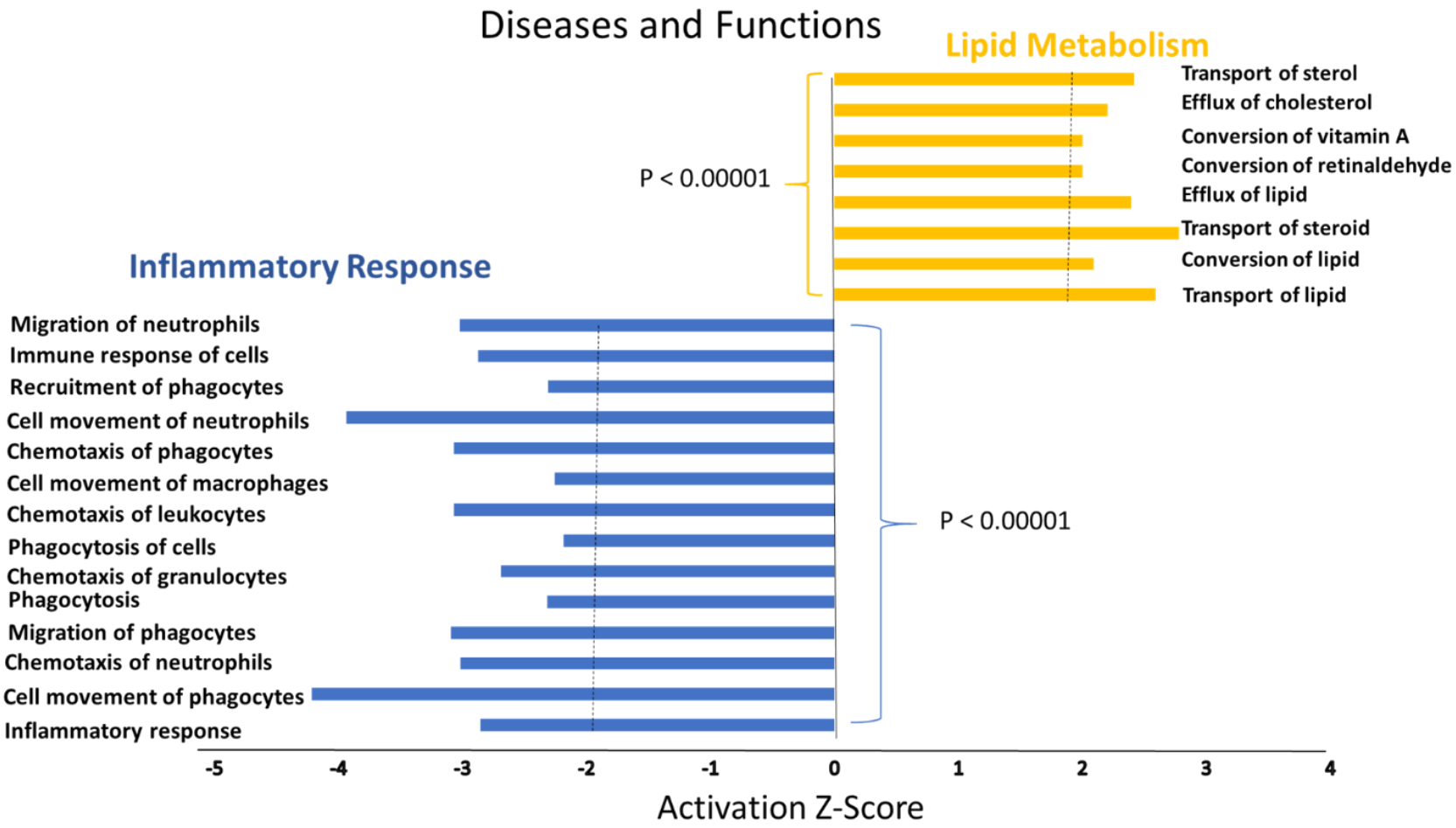


**Figure 3.** Specific functions predicted to be activated associated with lipid metabolism (orange) or predicted to be inhibited (blue) associated with inflammation in the liver of obese Zucker rats provided diets containing SPI-based diet compared to those provided a CAS-based diet for 16 weeks.

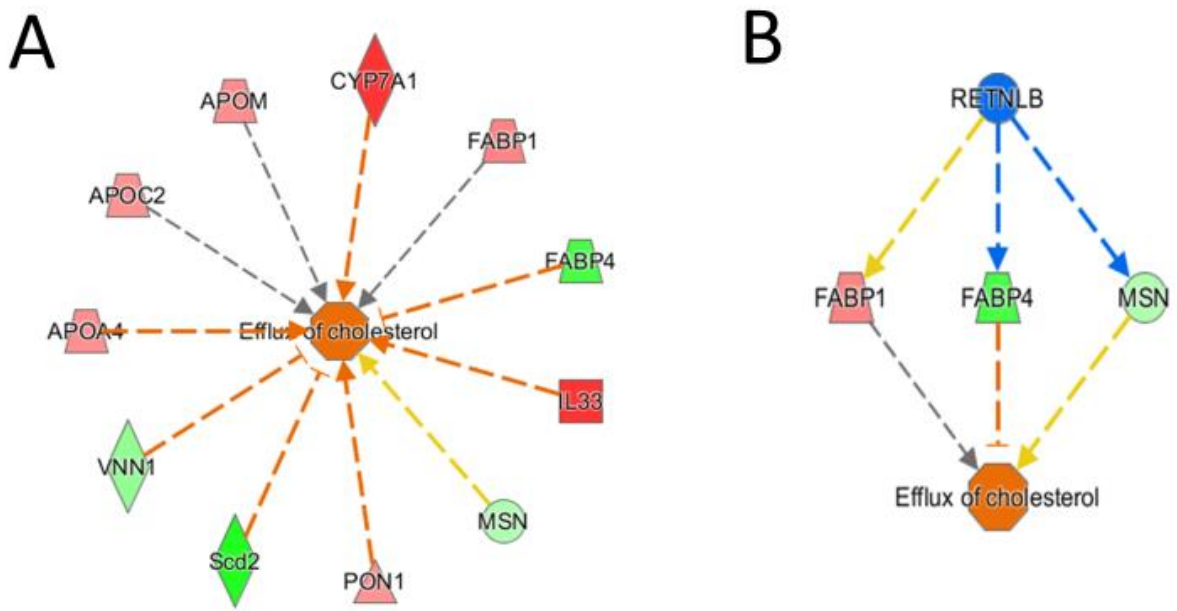


**Figure 4.** Gene expression networks leading to prediction of enhanced efflux of cholesterol in the RNAseq data in liver of 16 wk old obese rats fed CAS- and SPI-based diets. (A) Network of gene expression in the dataset used to calculate the activation Z score of efflux of cholesterol. Genes in pink or red were upregulated in the SPI-fed rats whereas those in green were down-regulated in SPI- vs CAS-fed rats. Genes and their individual fold difference expression values are; APOA4 (apolipoprotein A4, 1.37), APOC2 (apolipoprotein C2, 1.36), APOM (apolipoprotein M, 1.38), cytochrome P450 family 7 subfamily A, member 1,1.98), FABP1 (fatty acid binding protein 1, 1.41, FABP4 (fatty acid binding protein 4, -1.91), IL33, (Interleukin 33, 2.32), MSN (moesin,-1.32), PON1 (paraoxanase 1, 1.35), Scd2 (stearoyl-Coenzyme A desaturase 2, -2.42), VNN1, (vanin 1, -1.46). (B) This is a regulatory network that predicts that RETNLB (restin like protein beta) transcription factor would be predicted to be inhibited in the SPI-fed rats.

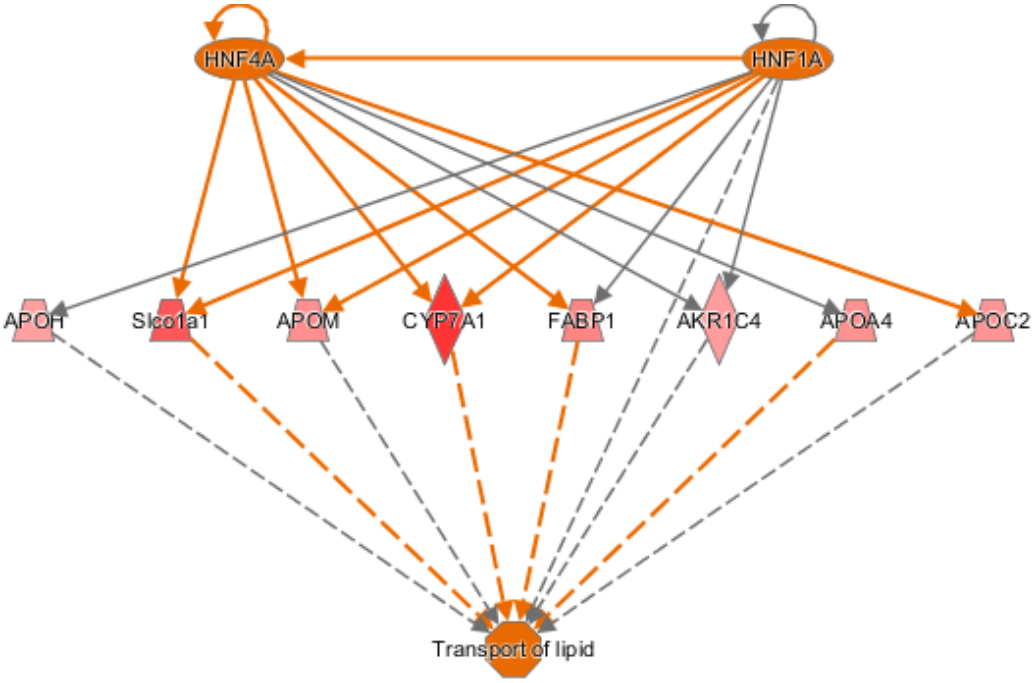


**Figure 5.** Regulator network for transport of lipid with two upstream regulators. The hepatocyte nuclear factors one (HNF1) homeobox A (HNF1A) and HNF1 factor 4 alpha (HNF4A) are predicted to be activated and enhance lipid transport in SPI-fed rats through the network of genes shown in the figure that include: APOH (apolipoprotein H, 1.32), Slco1a1 (solute carrier organic ion transporter family member 1A1, 1.62), APOM (apolipoprotein M, 1.38), CYP7A1 (cytochrome P450 family 7 subfamily A member 1), FABP1 (fatty acid binding protein 1, 1.42), AKR1C4 (also-keto reductase family member c14, 1.45), APOA4 (apolipoprotein A4, 1.37), and APOC2 (apolipoprotein C2, 1.36).

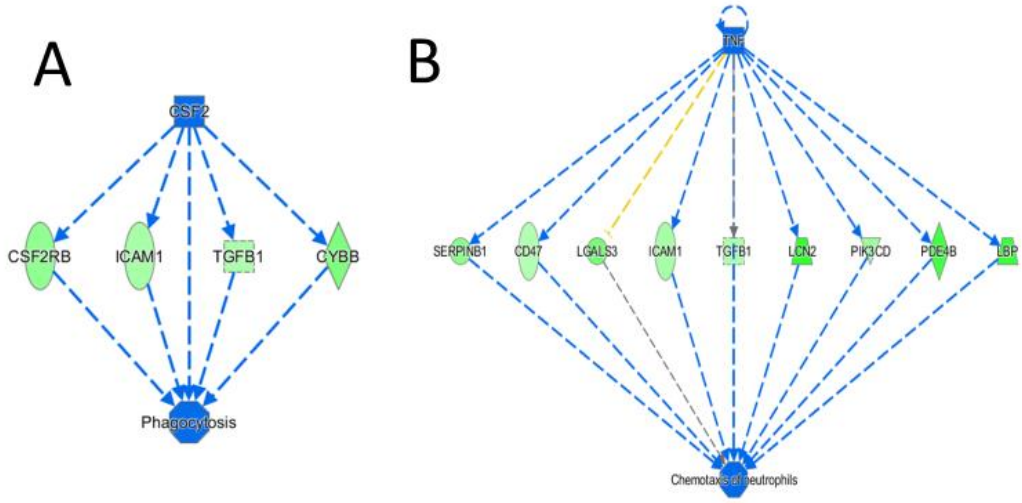


**Figure 6.** Regulator networks associated with specific components of inflammatory response of phagocytosis (A) and chemotaxis of neutrophils (B) that were predicted to be inhibited in SPI-fed compared to CAS-fed rats. In (A) the transcription factor colony stimulating factor 2 (CSF2) was predicted to be in inhibited in SPI-fed rats based on downregulation of CSF2RB, ICAM1, TGFB1 and CYBB. In (B), TNF (tumor necrosis factor) was predicted to be inhibited in SPI-fed rats based on the expression of genes shown in the network. Genes and fold differential expression in the regulatory networks are: CSFR2B (colony stimulating factor 2 receptor 2 beta common subunit, -1,47), ICAM1 (intracellular adhesion molecule 1, -1.36), TGFB1 (transforming growth factor beta 1, -1.33), CYBB (cytochrome b-245 beta chain, -1.57), SERPINB1 (serpin family B member 1, -1.50), CD47 (CD47 molecule, -1.30), LGALS3 (glactin 3, -1.62), LCN2 (lipocalin 2, -2.10), PIK3CD (phosphoinositol-4,5 bisphoaste 3 kinase catalytic subunit D, -1.41), PDE4B (phosphodiesterase 4B, -1.93), and LBP (lipopolysaccharide binding protein, -1.99).

**Supplementary Table 1.** Upstream regulators predicted to be inhibited (A) or activated (B) in the liver after 16 weeks in SPI-fed compared to CAS-fed Obese male Zucker rats.

A

| Upstream Regulator | Name | Molecule Type | Activation z-score |
|---|---|---|---|
| TNF | Tumor necrosis factor | Cytokine | -3.37 |
| IFNG | Interferon Gamma | Cytokine | -3.30 |
| ERK | MAPK group | Group | -3.11 |
| PRL | Prolactin (member growth hormone) | Cytokine | -2.77 |
| ERBB2 | Member epidermal growth factor receptor | Kinase | -2.76 |
| Pkc(s) | Protein Kinase C | Group | -2.68 |
| MKNK1 | MAPK interacting kinase 1 | Kinase | -2.65 |
| MYD88 | MYD88 inate immune signal transducer adaptor | Other | -2.57 |
| VEGFA | Vascular endothelial growth factor A | growth factor | -2.48 |
| TP53 | Tumor protein p53 | transcription regulator | -2.42 |
| NFkB | Nuclear Factor kappa B | Complex | -2.37 |
| SYVN1 | Synoviolin 1 | Transporter | -2.33 |
| RAC1 | RAC family small GTPase | enzyme | -2.32 |
| IL1B | Interleukin 1 beta | cytokine | -2.32 |
| CSF2 | Colony Stimulating Factor 2 | cytokine | -2.29 |
| Jnk | Jnk dimer | group | -2.28 |
| IL1A | Interleukin 1 A | cytokine | -2.25 |
| TGM2 | Transglutaminase 2 | enzyme | -2.24 |
| CD44 | CD44 molecule | other | -2.24 |
| LTBR | Lymphotoxin beta receptor | transmembrane receptor | -2.24 |
| PLG | Plasminogen | peptidase | -2.22 |
| GLI1 | GLI family zinc finger 1 | transcription regulator | -2.22 |
| MAP3K1 | Mitogen activated protein kinase kinase kinase 1 | kinase | -2.20 |
| FGF19 | Fibroblast growth factor 19 | growth factor | -2.19 |
| EZH2 | Enhancer of zeste 2 polycomb repressive complex 2 | transcription regulator | -2.18 |
| RETNLB | Restin like beta | other | -2.14 |
| IL5 | Interleukin 5 | cytokine | -2.02 |
| Pka | Protein kinase alpha | complex | -2.00 |
| TNFRSF1B | Tumor necrosis factor receptor 1 beta | transmembrane receptor | -2.00 |
| PPP1R13L | Protein phosphatase 1 regulatory subunit 13 like | transcription regulator | -2.00 |
| RUNX2 | RUNX family transcription factor 2 | transcription regulator | -2.00 |
| CNR1 | Cannaboid Receptor 1 | G-protein coupled receptor | -2.00 |

**Supplementary Table 2.** Upstream regulators predicted to be inhibited (A) or activated (B) in the liver after 16 weeks in SPI-fed compared to CAS-fed Obese male Zucker rats. (Cont.)

B

| Upstream Regulator | Name | Molecule Type | Activation z-score |
|---|---|---|---|
| ACOX1 | Acyl-CoA oxidase 1 | enzyme | 4.04 |
| HNF4A | Hepatocyte nuclear factor 4 alpha | transcription regulator | 3.44 |
| PKD1 | Polycystin 1, transient receptor potential channel interacting | ion channel | 2.89 |
| INSIG1 | Insulin induced gene 1 | other | 2.71 |
| SNAI1 | Snail family transcriptional repressor 1 | transcription regulator | 2.56 |
| HNF1A | Hepatocyte nuclear factor 1 homeobox A | transcription regulator | 2.55 |
| ABCB6 | ATP binding cassette subfamily B member 6 | transporter | 2.39 |
| ABCB4 | ATP binding cassette subfamily B member 4 | transporter | 2.22 |
| α catenin | CTNN alpha | group | 2.18 |
| IgG | Immunoglobulin G | complex | 2.17 |
| AHR | Aryl hydrocarbon receptor | ligand-dependent nuclear receptor | 2.15 |
| TRIM24 | Tripartite motif containing 24 | transcription regulator | 2.12 |
| ZNF106 | Zinc finger protein 106 | other | 2.00 |
| GPS2 | G protein pathway suppressor 2 | transcription regulator | 2.00 |
| SAFB | Scaffold attachment factor beta | other | 2.00 |

**Chapter 5**

A Comparison of Short and Long-Term Soy Protein Isolate Intake and its ability to reduce Liver Steatosis in obese Zucker Rats through modifications of genes involved in inflammation and lipid transport

**5.1 Abstract**: Transcriptomics (RNAseq) was conducted on liver samples from male obese Zucker rats fed diets containing either soy protein isolate (SPI) or casein (CAS) for 8 and 16 weeks. Interpretation of the transcriptomic data was carried out using Ingenuity Pathway Analysis (IPA) software that enables predictions of activation or inhibition of transcription regulators as well as disease and metabolic functions to be made that is based on expression of downstream molecules in the dataset as well as relationships of molecules reported in the database of the IPA program. In this study, we compare and contrast the results obtained for short (8 weeks) and long (16 weeks) term feeding of the diet on functions and upstream regulators. We have focused on two main functions: inflammatory response and lipid metabolism. In inflammatory response, predicted to be inhibited in SPI feeding, we discussed the role of the superfamily of cytokines interleukin 1 (IL-1) in the development of NAFLD and how its inhibition could be contributing to the amelioration of liver steatosis. On the side of lipid metabolism, predicted to be activated in SPI feeding, we discussed the probable role of two controversial or still not well understood upstream regulators in relation to the development of NAFLD: TRIM24 and PKD1.

## 5.2. Introduction

Obesity is simply defined by extra-accumulation of fat in adipose tissues du to either excess of calory intake, reduce energy expenditure, or both. According to the Centers for Disease Control and Prevention (CDC), the prevalence of adults with obesity in the United States for the period 2017-2018 was of 42.4%; and it has tripled in the last 30 years. For these reasons it is considered and epidemic and an increasing public health issue (Kincaid, Nagpal, and Yadav 2020). Obesity is usually associated with insulin resistance, high content of cholesterol and triglycerides in blood, cardiovascular disease, diabetes type 2, and fatty liver disease (Niederreiter and Tilg 2018). Nonalcoholic fatty liver disease (NAFLD) is a liver condition in which the accumulation of lipids in the hepatocytes, and the consequent inflammation, cannot be explained by alcohol consumption (Castaño-Rodríguez et al., 2017; Moschen et al., 2013). The complete disorder progresses through a series of stages starting with the aggregation of lipids in the liver tissue or steatosis. Once the inflammation progresses and fibrosis appear the next stage is called nonalcoholic steatohepatitis (NASH), that ultimately leads to cirrhosis; 20% of these cases progresses towards hepatocarcinoma (HCC) (Castaño-Rodríguez et al. 2017; Moschen et al. 2013). The development of NAFLD is considered to be the result of complex and not well understood interactions between environmental and genetic factors, including the immune system and the gastrointestinal microbiome (Niederreiter and Tilg 2018).

There is evidence that dietary components, such as isoflavones and resveratrol, that can alleviate the symptoms of metabolic conditions such as liver steatosis (Chen et al., 2015). Resveratrol is a natural polyphenol found in grapes, peanuts, berries, and red wine. Currently, resveratrol is used as a dietary supplement. Resveratrol can regulate liver lipid metabolism to prevent the development of NAFLD in animals (Mukherjee, 2010). Another diet component, genistein is one of the most abundant isoflavones in soybean. It has been found that at the cellular level genistein inhibits cellular cholesterol synthesis and cholesterol esterification in

HepG2 human hepatoma cells (Borradaile et al, 2002). Genistein also affects fatty acid oxidation. It exerts antidiabetic and hypolipidemic effects through the upregulation of the PPAR-regulated (peroxisome proliferator-activated receptor) gene expression. Thus, the effects of genistein on cholesterol synthesis and fatty acid oxidation are well known. However, the effect of genistein on fatty acid synthesis has not yet been identified (Shin et al. 2007). Genistein has also been reported to enhance adipogenesis through modification of peroxisome proliferator-activated receptor-γ (PPARγ) (Relic et al. 2009) and canonical Wnt/β-catenin signaling (Su and Simmen 2009). Genistein could be controlling gene expression through DNA methylation (Johnson et al. 2012).

A soy protein isolate (SPI)-based diet was shown to attenuate liver steatosis in obese Zucker rats that was not due to increased arginine intake afforded by the soy protein isolate intake (Hakkak et al. 2015). Global gene expression analysis of liver has revealed insight into mechanisms by which SPI-based diet attenuated liver steatosis based on the most differentially expressed genes (up- and down-regulated) between SPI- and casein (CAS)-based diets (Kozaczek et al., 2019 [Chapter 3]) and upstream regulators and functions (Chapter 4) after 8 weeks of feeding the respective diets. The purpose of this study was to assess the effects of short-term (8 weeks) vs long-term (16 weeks) of feeding SPI- or CAS-based diets on the progression of NAFLD-related gene expression in this obese Zucker rat model.

## 5.3. Materials and Methods

Animal codes were authorized by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences (Protocol code number 3242; approved on 12/6/2011). Liver tissue was obtained from male obese Zucker rats from a previous study (Hakkak et al. 2015). 6 weeks old obese Zucker rats were purchased from Harlan Laboratories (Indianapolis, IN). After one week of acclimation, rats were randomly assigned to either a casein (CAS) diet or soy protein isolate (SPI) diet. They were housed in individual cages and provided

the diets ad libitum for 16 weeks and all rats were humanely killed, and liver samples obtained, and flash frozen in liquid nitrogen and stored at −80°C.

A phenol-chloroform solution was used to extract RNA from liver samples. A 1% agarose gel was used to evaluate the quality of RNA and concentrations were assessed with Take 3 micro volume plate with a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT). RNA sequencing (RNAseq) of RNA samples was conducted using an Illumina HiSeq 100 base pair paired end read at the Research Support Facility (Michigan State University, East Lansing, MI). The CLC Genomics Workbench 8 software that incorporates the pipeline recommended by Mortazavi et al. (2008) was used to map the reads to *Rattus rattus* genome assembly (version 4). The RPM data was transformed using log2 to stabilize the variance and then performed a further quantile normalization. Over 1200 transcripts were differentially expressed (> 1.3 fold difference and $P < 0.05$). Ingenuity Pathway Analysis (IPA) commercial software (Qiagen, CA) was used to help in the interpretation of the dataset.

The analysis of the mRNA expression data was assessed with the software Graph Pad Prism version 6.00 for Windows, La Jolla California USA, and Student's t-test. Differences were considered significant at $P<0.05$. Ingenuity Pathway Analysis software (Qiagen, CA) was used in the analysis of global gene expression data. This analysis includes predictions of activation or inhibition of upstream regulators and functions based on prior knowledge of expected effects between transcriptional regulators and their target genes from published citations stored in the IPA program. If the observed direction of change is mostly consistent with either activation or inhibition of the transcriptional regulator, then a prediction is made, and an activation z score generated that is also based on literature-derived regulation direction (i.e. "activating" or "inhibiting"). Activation z scores > 2.0 indicate that a molecule is activated whereas activation z scores of < -2.0 indicate that a target molecule is inhibited. The p-value of overlap measures whether there is a statistically significant overlap between the dataset molecules and those

regulated by an upstream regulator is calculated using Fisher's Exact Test, and significance is attributed to p-values < 0.05.

## 5.4. Results and Discussion

NAFLD has become a major public health issue worldwide. In this condition, the first signs are a detrimental aggregation of lipids within the liver cells and inflammation causing steatosis which can later develops fibrosis and NASH, to finally lead to cirrhosis and HCC (Fang et al. 2018). In this study, we compared the effects of short (8 weeks) and long-term (16 weeks) of feeding a SPI-diet that attenuated liver steatosis compared to rats fed a CAS-diet (Hakkak et al. 2015) on targeted gene expression, functions (inflammatory processes and lipid metabolism), and upstream regulators in liver from Obese Zucker rats rat model.

Previously, we presented the results of feeding SPI- and CAS-based diets for 8 weeks on targeted gene expression obtained by RNAseq and PCR in obese Zucker rats (Kozaczek et al. 2019). Only three out of 12 most differentially genes, NPTX2, IL33 and PRSS8, were still present among the most differentially expressed genes at 16 weeks in the RNA-seq dataset that were present at 8 weeks. The top genes that were differentially expressed at both 8 and 16 weeks are shown in Table 1. Only one gene (LCN2) exhibited a change from down regulation at 8 weeks to upregulation at 16 weeks in the SPI-diet group; the remaining genes exhibited similar direction of differential expression at both 8 and 16 weeks. The discussion of the roles these genes may play in liver steatosis is provided in Kozaczek et al. (2019, Chapter 3) and in Chapter 4 of this dissertation and will not be repeated here.

We observed an enhancement of the lipid metabolism and an expansion of decreased inflammatory response in SPI-diet in the liver going from 8 weeks to 16 weeks of consuming the diet compared to CAS-fed rats (Fig. 1). The longer the rats were treated with SPI the more beneficial the effects of this diet on the improvement of lipid metabolism, and constraining the acute inflammatory response observed in this condition (Fig. 1). It is remarkable how at 8 weeks of SPI diet there were only two functions of lipid metabolism predicted to be activated, while at

16 weeks of SPI feeding the number of functions involved in lipid metabolism predicted to be activated increased up to eight. Several functions that were predicted to be activated (e.g. lipid transport, efflux, and conversion of lipid) in SPI-fed rats are processes that could reduce lipid accumulation in hepatocytes. Similarly, processes and functions involved in inflammatory response such as phagocytosis, chemotaxis of neutrophils, and immune response of cells, were inhibited in SPI-fed rats thus ameliorating NAFLD symptoms. A wide range of molecules were predicted to be activated or inhibited in liver of obese rats fed an SPI- compared to CAS-based diet, as well as complete pathways. As discussed below, these predictions point towards mechanisms by which SPI ameliorates liver steatosis in the long-term and can serve as hypotheses to be tested in future studies.

The inflammatory response triggered during NAFLD is still not fully understood. Several key molecules that were predicted to be inhibited in the liver of the SPI-fed rats known to be closely involved in inflammation are presented in Table 2. We have covered some of these target molecules or upstream regulators in previous chapters, such us TNF, CSF2 and its receptor CSF2RB, TNFRSF12A, and LCN2. However, in this chapter we start to see how all these molecules and intricate pathways are at last intertwined. Moreover, we start to observe the beneficial consequences of a sustained SPI diet over 16-week time period (Figure 1 and Table 2).

On the side of inflammation and immune response in the liver, high levels of fatty acids cause insulin resistance and lipotoxicity, and in addition to other components can promote a cascade of inflammation (Fang et al. 2018). The primary pro-inflammatory cytokines related with the development of NAFLD and progression to NASH are interleukin 1 (IL-1) family, tumor necrosis factor (TNF) and IL-6 (Fang et al. 2018; Niederreiter and Tilg 2018). It is important to highlight that these three key types of cytokines are also expressed by adipose tissue in the presence of inflammation (Niederreiter and Tilg 2018). TNF was already discussed in Chapter 4,

although such a conspicuous strong immune mediator it is also present in the current Chapter for a comparison between short and long-term SPI diets (Table 2). TNF was predicted to be inhibited in SPI feeding at both short and long-term diet, with a z-score value of -4.22 at 8 weeks and -3.37 for 16 weeks of feeding, respectively. In other words, TNF had a very consistent inhibition during the entire time on SPI diet, which could account in part for the mitigation of liver steatosis.

The IL-1 superfamily of cytokines includes a wide repertoire of members, their receptors, and antagonists. This family includes IL-1A, IL-1B, IL-1R antagonist, IL-18, IL-33, IL-36, IL-37, IL-38 (Mirea et al. 2018; Tilg et al. 2020), their receptors with simplified nomenclature from IL-R1 to IL-R10, and decoy receptors (Garlanda et al. 2013). IL-1 cytokines are present in almost all tissues and organs in the body, and as with most cytokines, they are described as having pleiotropic biological functions (Tilg et al. 2020; Garlanda et al. 2013). Decoy receptors evolved as a way to tightly control the devastating inflammatory reactions IL-1 can exert upon their target site (Garlanda et al. 2013). Some of these cytokines are pro-inflammatory, but some others can behave as anti-inflammatories (Tilg et al. 2016). IL-33 was discussed in Chapter 3, as an important “alarmin” of the body’s immune response. Some of the other IL-1 type of cytokines will be discussed below. IL-1A and IL-1B were predicted to be inhibited in SPI treated rats, with z-scores values of -2.59 at 8 weeks and -2.25 at 16 weeks for IL-A, and -3.53 at 8 weeks and -2.32 at 16 weeks for IL-B, respectively (Table 2).

Whereas IL-1A is released in an active form from already damaged necrotic and apoptotic hepatocytes, IL-1B is released in a pro-active form that requires further activation (Mirea et al. 2018). IL-1A promotes the activation of a cytokines cascade conducing to sterile inflammation, such as in NAFLD, which is inflammation without external mediators such as bacteria and endotoxins (Tilg et al. 2016). IL-1B needs to be activated via interaction with inflammasomes. Inflammasomes are multiprotein complexes constituted by caspases and

elements acquired from injured cells and microbes (Buzzetti et al. 2016). Once the pro-IL-1B is cleaved by caspases within the inflammasome it is finally in its active form. IL-1B stimulates the aggregation of triglycerides in the hepatocytes and formation of lipid droplets. In addition, IL-1B promotes the production of IL-6 (predicted to be inhibited in this study at 8 weeks, z-score = -2.34, Table 2), another pro-inflammatory cytokine closely involved in the development of NAFLD and NASH simultaneously with TNF (Mirea et al. 2018). It has been reported that IL-1B can suppress the expression of IL-33 during intestinal helminth infection (Zaiss et al. 2013). An important difference between IL-1A and IL-1B is their cellular location. While IL-1A is usually localized in the nucleus acting as a transcription factor, IL-1B is mainly located in the cytosol (Garlanda et al. 2013). The type of death of a hepatocyte also influences the type of immune response IL-1A will trigger. During necrosis, IL-1A is released to the medium and unleashes a neutrophilic inflammation; in an apoptotic cell the IL-1A remains chromatin-bound and it is not available for the same inflammation pathway (Garlanda et al. 2013). It appears that SPI may attenuate liver steatosis by inhibiting IL-1A and IL-1B.

IL-18 is predicted to be inhibited at 8 weeks, z-score -2,19, but not in rats receiving the SPI diet for 16 weeks (Table 2). Although IL-18 is also activated by the inflammasome and has also been studied in relation with metabolic disorders and NAFLD, the results in animal models compared to those in humans are inconsistent (Mirea et al. 2018). Mice with IL-18 deficiency presented signs of metabolic syndrome, such as dyspilidemia, and insulin resistance, that suggests there may be a protective role of this cytokine against NAFLD (Netea et al. 2006; Yamanishi et al. 2016). However, studies in humans have correlated high plasma levels of IL-18 with insulin resistance, type 2 diabetes and atherosclerosis suggesting that IL-18 could be involved in the progression of metabolic disorders (Hung et al. 2005; Trøseid et al. 2010). The predicted inhibition of IL-18 by SPI diet in our study suggests similarities with the mouse studies (Netea et al. 2006; Yamanishi et al. 2016) but not those in humans (Hung et al. 2005; Trøseid et

al. 2010). Further research on IL-1A and B and its role in liver steatosis and NAFLD is warranted.

Upstream regulators related with the improvement of lipid metabolism in SPI feeding such as Acyl-CoA oxidase 1 (ACOX1) and the transcription factors Hepatocyte nuclear factor 1 and 4 alpha (HNF1A and HNF4A, respectively) were discussed in Chapter 4. Here we can follow these genes in both short and long-term diet with SPI (Table 3). ACOX1 was predicted to be active at 8 weeks (z-score = 3.12) and at 16 weeks (z-score = 4.04). HNF1A was activated at both 8 and 16 weeks (z-scores = 2.24 and 2.55). Interestingly, HNF4A was only predicted to be active at 16 weeks of SPI feeding (z-score = 3.44). Since both transcription factors regulate the expression of the other, it is not clear why HNF4A was not predicted to be activated after 8 weeks in the present study.

Tripartite Motif Containing 24 (TRIM24) regulates gene expression by binding to the activation function 2 (AF2) region of several nuclear factors such as estrogen, retinoic acid, and vitamin D3 receptors (Charrez et al. 2016). TRIM24 is predicted to be activated at 8 and 16 weeks with z-scores values of 2.63 and 2.12, respectively (Table 3). Similar to findings on IL-1A and 1B above, there are discrepancies in studies of this TRIM24 in animal models compared to those in humans. In murine models the deficiency or lack of TRIM24 is associated with the decrease in expression of genes involved in oxidation and reduction, lipid and steroid metabolism, and the increase of genes involved in endoplasmic reticulum stress as well as cell cycle pathways; all of which are characteristics associated with NAFLD (Gu et al. 2016). Its normal expression is linked to the repression of lipid accumulation in liver cells (Jiang et al. 2015). The complete loss of function of TRIM24 can drive the progression of NAFLD to NASH to HCC (Jiang et al. 2015). Conversely, in humans the expression of TRIM24 is associated with the development of different types of cancer, including HCC (Freese et al. 2019). Our study

concurs with previous studies base on mice and rat models, since TRIM24 seems to be helping with the reduction of liver steatosis in animals consuming the SPI-based diet.

Polycystin 1 (PKD1), jointly with PKD2, is part of a receptor ion channel complex that when mutated causes polycistic kidney disease (Menezes et al. 2016; Roy and Marin 2018). PKD1 was predicted to be activated at 8 weeks with z-score of 2.40, and at 16 weeks with a z-score of 2.89, respectively (Table 3). PKD1 has been extensively studied and linked with polycystic kidney disease and polycystic liver disease (Cnossen and Drenth 2014; Roy and Marin 2018). However, to our knowledge only one study has associated this molecule with NAFLD (Rada et al. 2018).

In summary, it is apparent that feeding a SPI-based diet to a genetically obese male rat model that reduced liver steatosis resulted in predicted reductions (based on expression of genes in the dataset and literature citations in the pathway analysis database) in inflammatory mechanisms while enhancing lipid transport out of the liver. Around each of the upstream regulators that are presented in the Tables 2 and 3 predicted to be activated or inhibited in this transcriptomic dataset, hypotheses can be generated and tested in future studies to help develop treatments that can alleviate liver steatosis and hopefully slow the progression of NAFLD to NASH and cirrhosis.

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5. 5 Tables and Figures

**Table 1.** Top differentially expressed genes that up and down-regulated (out of the top 10) that were differentially expressed at both 8 and 16 weeks in rats fed the SPI-diet compared to those fed the CAS-diet. Mean values (n=8)

| | Fold Diff RNAseq | |
|---|---|---|
| Genes | 8 weeks | 16 weeks |
| NPTX2 | 1.88 | 3.8 |
| GPT | 2.02 | 1.60 |
| INMT | 1.83 | 1.5 |
| HAL | 1.85 | 1.95 |
| Serpina6 | 1.51 | 1.36 |
| IL33 | 1.52 | 2.32 |
| PRSS8 | -5.71 | -3.09 |
| Ajuba | -2.65 | -2.81 |
| CSF2RB | -1.46 | -2.69 |
| CYP2C12 | -2.45 | -2.3 |
| LCN2 | -2.45 | 1.39 |
| TNFRSF12A | -2.3 | -1.81 |

**Table 2.** Comparison of Upstream regulators predicted to be inhibited after 8 and 16 weeks of SPI feeding (short and long-term diet). The numbers between the 8 and 16 week upstream regulator predictions show the coincidence of predicted inhibition of genes (in rank order of inhibition for 8 vs 16 weeks and 16 vs 8 weeks. (An 'x' indicates that the prediction was not provided at 16 weeks, and a 'y' indicates that the prediction was not provided at 8 weeks). Activation Z-scores all had a P value of overlap (Fisher's exact t-test) of $P < 0.01$.

| 8 weeks | | | | | | 16 weeks | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| Upstream Regulator | Name | Molecule Type | Activation z-score | 8 vs 16 wk | 16 vs 8 wk | Upstream Regulator | Name | Molecule Type | Activation z-score |
| 1. IFNG | Interferon gamma | cytokine | -4.94 | 1-2 | 1-2 | 1. TNF | Tumor necrosis factor | Cytokine | -3.37 |
| 2. TNF | Tumor necrosis factor | cytokine | -4.22 | 2-1 | 2-1 | 2. IFNG | Interferon Gamma | Cytokine | -3.30 |
| 3. IL1B | Interleukin 1 beta | cytokine | -3.53 | 3-14 | 3-17 | 3. ERK | MAPK group | Group | -3.11 |
| 4. MYD88 | Myeloid differentiation primary response 88 | other | -3.51 | 4-8 | 4-6 | 4. PRL | Prolactin (member growth hormone) | Cytokine | -2.77 |
| 5. NFkB (complex) | Nuclear factor kappa enhancer of B cells | complex | -3.22 | 5-11 | 5-y | 5. ERBB2 | Member epidermal growth factor receptor | Kinase | -2.76 |
| 6. PRL | Prolactin | cytokine | -3.22 | 6-4 | 6- | 6. Pkc(s) | Protein Kinase C | Group | -2.68 |
| 7. UCP1 | Uncoupling Protein 1 (Thermogenin) | transporter | -3.06 | 7-x | 7-31 | 7. MKNK1 | MAPK interacting kinase 1 | Kinase | -2.65 |
| 8. CHUK | Inhibitor of nuclear factor kappa-B kinase | kinase | -2.97 | 8-x | 8-4 | 8. MYD88 | MYD88 innate immune signal transducer adaptor | Other | -2.57 |
| 9. TICAM1 | Protein Inducing Interferon Beta | other | -2.84 | 9-x | 9-20 | 9. VEGFA | Vascular endothelial growth factor A | growth factor | -2.48 |
| 10. RETNLB | Resistin-like beta | other | -2.83 | 10-26 | 10-x | 10.TP53 | Tumor protein p53 | transcription regulator | -2.42 |
| 11. TLR4 | Toll-like receptor 4 | transmembrane receptor | -2.78 | 11-x | 11-5 | 11. NFkB | Nuclear Factor kappa B | Complex | -2.37 |
| 12. IKBKB | Inhibitor of Nuclear Factor Kappa B Kinase beta | kinase | -2.74 | 12-x | 12-y | 12. SYVN1 | Synoviolin 1 | Transporter | -2.33 |
| 13. CG | Vitamin D Binding Protein | complex | -2.65 | 13-x | 13-y | 13. RAC1 | RAC family small GTPase | enzyme | -2.32 |
| 14. FGF2 | Fibroblast Growth Factor 2 | growth factor | -2.65 | 14-x | 14-3 | 14. IL1B | Interleukin 1 beta | cytokine | -2.32 |
| 15. CD44 | Phagocytic Glycoprotein 1 | other | -2.62 | 15- | 15-22 | 15. CSF2 | Colony Stimulating Factor 2 | cytokine | -2.29 |
| 16. Pkc(s) | Protein Kinase C | group | -2.61 | 16- | 16-27 | 16. Jnk | Jnk dimer | group | -2.28 |
| 17. ERK | MAPK group | group | -2.61 | 17-3 | 17-19 | 17. IL1A | Interleukin 1 A | cytokine | -2.25 |
| 18. EGFR | Epidermal growth factor receptor | kinase | -2.59 | 18-x | 18-y | 18. TGM2 | Transglutaminase 2 | enzyme | -2.24 |
| 19. IL1A | Interleukin 1 alpha (hematopoietin 1) | cytokine | -2.59 | 19-17 | 19-15 | 19. CD44 | CD44 molecule | other | -2.24 |
| 20. VEGFA | Vascular endothelial growth factor A | growth factor | -2.48 | 20-9 | 20-y | 20. LTBR | Lymphotoxin beta receptor | transmembrane receptor | -2.24 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 21. ERK1/2 | Mitogen-Activated Protein Kinase 3 | group | -2.43 | 21-x | 21-y | 21. PLG | Plasminogen | peptidase | -2.22 |
| 22. CSF2 | Colony Stimulating Factor 2 | cytokine | -2.43 | 22-15 | 22-y | 22. GLI1 | GLI family zinc finger 1 | transcription regulator | -2.22 |
| 23. GNA12 | G Protein Subunit Alpha 12 | enzyme | -2.41 | 23-x | 23- | 23. MAP3K1 | Mitogen activated protein kinase kinase kinase 1 | kinase | -2.20 |
| 24. CD38 | Cyclic ADP-Ribose Hydrolase 1 | enzyme | -2.41 | 24-x | 24-y | 24. FGF19 | Fibroblast growth factor 19 | growth factor | -2.19 |
| 25. IRF3 | Interferon regulatory factor 3 | transcription regulator | -2.36 | 25-x | 25-y | 25. EZH2 | Enhancer of zeste 2 polycomb repressive complex 2 | transcription regulator | -2.18 |
| 26. P38 MAPK | P38 MAP Kinase | group | -2.35 | 26-x | 26-10 | 26. RETNLB | Restin like beta | other | -2.14 |
| 27. Jnk | Jnk dimer group | group | -2.35 | 27-16 | 27-y | 27. IL5 | Interleukin 5 | cytokine | -2.02 |
| 28. IL6 | Interleukin 6 | cytokine | -2.34 | 28-x | 28-y | 28. Pka | Protein kinase alpha | complex | -2.00 |
| 29. IL4 | Interleukin 4 | cytokine | -2.31 | 29-x | 29-30 | 29. TNFRSF1B | Tumor necrosis factor receptor 1 beta | transmembrane receptor | -2.00 |
| 30. TNFRSF1B | Tumor necrosis factor receptor 1 beta | transmembrane receptor | -2.24 | 30-29 | 30-y | 30. PPP1R13L | Protein phosphatase 1 regulatory subunit 13 like | transcription regulator | -2.00 |
| 31. MKNK1 | MAPK interacting kinase 1 | kinase | -2.24 | 31-x | 31-y | 31. RUNX2 | RUNX family transcription factor 2 | transcription regulator | -2.00 |
| 32. TLR7 | Toll-like receptor 7 | transmembrane receptor | -2.22 | 32-x | 32-y | 32. CNR1 | Cannaboid Receptor 1 | G-protein coupled receptor | -2.00 |
| 33. PRKCE | Protein kinase C epsilon | kinase | -2.22 | 33-x | | | | | |
| 34. CREB1 | Cyclic AMP-Responsive Element-Binding Protein 1 | transcription regulator | -2.22 | 34-x | | | | | |
| 35. IRF7 | Interferon regulatory factor 7 | transcription regulator | -2.21 | 35-x | | | | | |
| 36. F7 | Coagulation Factor VII | peptidase | -2.20 | 36-x | | | | | |
| 37. IL18 | Interleukin 18 | cytokine | -2.19 | 37-x | | | | | |
| 38. SIRT1 | Sirtuin 1 | transcription regulator | -2.18 | 38-x | | | | | |
| 39. Ifnar | Interferon Alpha/Beta Receptor | group | -2.13 | 39-x | | | | | |
| 40. STAT1 | Signal transducer and activator of transcription 1 | transcription regulator | -2.03 | 40-x | | | | | |
| 41. EGR1 | Early Growth Response 1 | transcription regulator | -2.00 | 41-x | | | | | |
| 42. HRG | Histidine Rich Glycoprotein | other | -2.00 | 42-x | | | | | |

**Table 3.** Comparison of Upstream regulators predicted to be activated after 8 and 16 weeks of SPI diet (short and long-term feeding). The numbers between the 8- and 16-week upstream regulator predictions show the coincidence of predicted inhibition of genes (in rank order of inhibition for 8 vs 16 weeks and 16 vs 8 weeks. (An 'x' indicates that the prediction was not provided at 16 weeks, and a 'y' indicates that the prediction was not provided at 8 weeks). All activation Z-scores had a P value of overlap (Fisher exact t-test) of $P < 0.001$.

| 8 weeks | | | | | | 16 weeks | | | |
|---|---|---|---|---|---|---|---|---|---|
| Upstream Regulator | Name | Molecule Type | Activation z-score | 8 vs 16 wk | 16 vs 8 wk | Upstream Regulator | Name | Molecule Type | Activation z-score |
| 1. ACOX1 | Acyl-CoA oxidase 1 | enzyme | 3.12 | 1-1 | 1-1 | 1. ACOX1 | Acyl-CoA oxidase 1 | enzyme | 4.04 |
| 2. α catenin | Alpha catenin | group | 2.87 | 2-9 | 2-y | 2. HNF4A | Hepatocyte nuclear factor 4 alpha | transcription regulator | 3.44 |
| 3. IgG | Immunoglobulin G | complex | 2.71 | 3-10 | 3-9 | 3. PKD1 | Polycystin-1 | ion channel | 2.89 |
| 4. VCAN | Versican | other | 2.65 | 4-x | 4-5 | 4. INSIG1 | Insulin induced gene 1 | other | 2.71 |
| 5. INSIG1 | Insulin induced gene 1 | other | 2.65 | 5-4 | 5-y | 5. SNAI1 | Snail family transcriptional repressor 1 | transcription regulator | 2.56 |
| 6. TRIM24 | Tripartite motif containing 24 | transcription regulator | 2.63 | 6-x | 6-13 | 6. HNF1A | Hepatocyte nuclear factor 1 homeobox A | transcription regulator | 2.55 |
| 7. COL18A1 | Endostatin | other | 2.45 | 7-x | 7-y | 7. ABCB6 | ATP binding cassette subfamily B member 6 | transporter | 2.39 |
| 8. HOXD10 | Homeobox D10 | transcription regulator | 2.45 | 8-x | 8-y | 8. ABCB4 | ATP binding cassette subfamily B member 4 | transporter | 2.22 |
| 9. PKD1 | Polycystin-1 | ion channel | 2.40 | 9-3 | 9-2 | 9. α catenin | Alpha catenin | group | 2.18 |
| 10. IL1RN | Interleukin 1 Receptor Antagonist | cytokine | 2.36 | 10-x | 10-3 | 10. IgG | Immunoglobulin G | complex | 2.17 |
| 11. mir-21 | MicroRNA 21 | microRNA | 2.31 | 11-x | 11-y | 11. AHR | Aryl hydrocarbon receptor | ligand-dependent nuclear receptor | 2.15 |
| 12. IL10RA | Interleukin 10 Receptor Alpha | transmembrane receptor | 2.27 | 12-x | 12-6 | 12. TRIM24 | Tripartite motif containing 24 | transcription regulator | 2.12 |
| 13. HNF1A | Hepatocyte nuclear factor 1 homeobox A | transcription regulator | 2.24 | 13-6 | 13-19 | 13. ZNF106 | Zinc finger protein 106 | other | 2.00 |
| 14. ZBTB20 | Zinc Finger and BTB Domain Containing 20 | transcription regulator | 2.24 | 14-x | 14-y | 14. GPS2 | G protein pathway suppressor 2 | transcription regulator | 2.00 |
| 15. IGHM | Immunoglobulin Heavy Constant Mu | transmembrane receptor | 2.21 | 15-x | 15-y | 15. SAFB | Scaffold attachment factor beta | other | 2.00 |
| 16. CFTR | Cystic Fibrosis Transmembrane Conductance Regulator | ion channel | 2.21 | 16-x | | | | | |
| 17. PSEN1 | Presenilin 1 | peptidase | 2.18 | 17-x | | | | | |
| 18. APOE | Apolipoprotein E | transporter | 2.12 | 18-x | | | | | |
| | | | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 19. ZNF106 | Zinc finger protein 106 | other | 2.00 | 19-13 | | | | | |
| 20. ACKR2 | Atypical Chemokine Receptor 2 | G-protein coupled receptor | 2.00 | 20-x | | | | | |
| 21. PRDM1 | PR Domain Zinc Finger 1 | transcription regulator | 2.00 | 21-x | | | | | |
| 22. CHD4 | Chromodomain-helicase-DNA-binding protein 4 | enzyme | 2.00 | 22-x | | | | | |
| 23. Mt2 | Metallothionein-2 | other | 2.00 | 23-x | | | | | |
| 24. Irgm1 | Immunity Related GTPase M | other | 2.00 | 24-x | | | | | |
| 25. Mt1 | Metallothionein-1A | other | 2.00 | 25-x | | | | | |

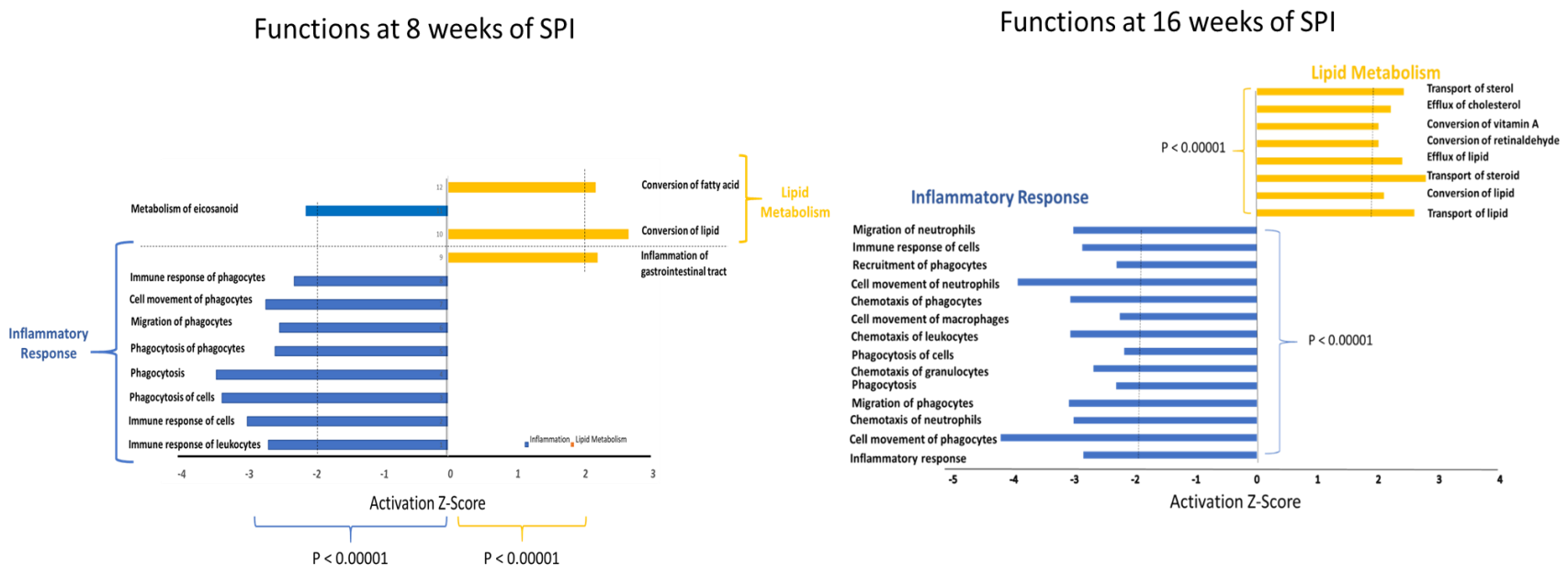


**Figure 1.** Comparison of Diseases and Functions related with Lipid metabolism predicted to be activated (orange) and Inflammatory response predicted to be inhibited (blue) at 8 and 16 weeks of SPI feeding. The number of functions inhibited and activated increased in the long-term SPI diet.

## CONCLUSION

In summary, we believe that the global gene expression analysis conducted in this study on liver tissue obtained by Hakkak et al. (2015) from genetically obese Zucker rats fed a diet containing Soy Protein Isolate versus Casein for 8 and 16 weeks (short- and long-term, respectively) has helped to gain some insight on the overall onset of non-alcoholic fatty liver disease and its interaction with diet components. In addition, we consider that the validation of the transcriptomic data was satisfactory, and that targeted gene expression has helped to understand the effects of different dietary protein sources (soy protein isolate versus a casein-based diet) on various cellular mechanisms. Furthermore, the soy protein isolate appears to reduce the expression of proinflammatory molecules/processes and to enhance lipid transport, mostly in the long-term, although can also be observed at 8 weeks. Conversely, casein seems to increase inflammatory processes, which concurs with evidence in literature. The upstream regulator analysis conducted with the commercial software program used in interpretation of the transcriptomic data has revealed potential insight into mechanistic networks associated with feeding soy protein isolate and casein based diets that requires further research to clearly elucidate these mechanisms. We consider that the results from this study can help not only in understanding the development of liver steatosis in obese animals, but also possibly lead to interventions that can help to prevent development of non-alcoholic steatohepatitis and irreversible damage to liver in the form of cirrhosis and development of hepatocellular carcinoma.